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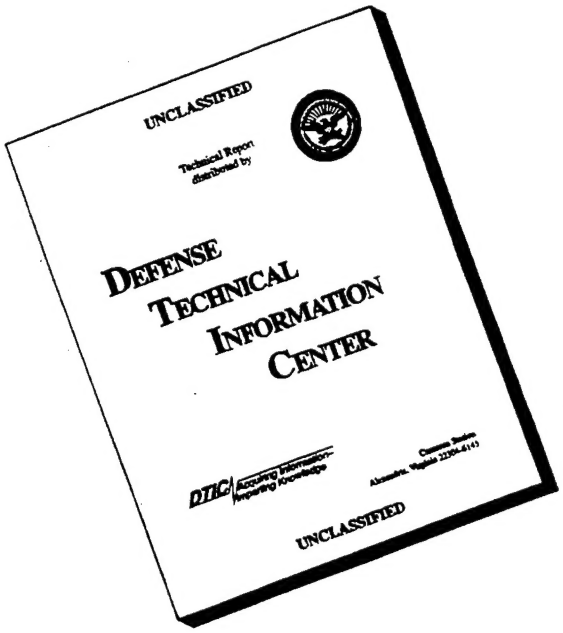

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Maria T. Bissell* Oct. 6, 1995

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ABSTRACT

By a three-dimensional reconstituted basement membrane (EHS matrix) assay, we have previously shown that breast morphogenesis is regulated by cell-extracellular matrix (ECM) interactions and these contacts are impaired in malignancy. A series of studies were conduced to investigate the significance of cell/ECM interactions in tumorigenesis. ECM influences expression of lactoferrin, a differentiation marker, either by altering cell-cell interactions or by providing mechanochemical signals that regulate cell shape. Emphasis on the importance of intact β1 integrin signalling was indicated by function blocking studies. Phenotypically normal HMT-3522 cells failed to grow or differentiate and underwent apoptosis in the presence of β1 integrin blocking antibodies. In contrast a number of tumorigenic cell lines demonstrated refractoriness to these effects indicating they have circumvented this regulatory pathway. Finally the importance of cell/ECM interactions in the maintenance of the differentiated phenotype was underscored by examining the effect of overexpression of the putative tumor suppressor gene nm23-H1 in metastatic cell line MDA-MB-435, where several aspects of normal breast morphogenesis was recapitulated. Further studies are presently underway to dissect other ECM components and their signalling pathways that are critical in breast cancer progression.

INTRODUCTION

Human breast cancer is thought to derive from the stepwise transformation of the luminal epithelial cells of the ducts and terminal lobular units (1,2). This has led to the conclusion that mutational events are critical to the genesis of the transformed phenotype. Mutations studied include the amplification or aberrant expression of the proto-oncogenes: c-*myc*, c-*erb*B2, *int*-2/*hst*-1 and infrequently H-*ras*, as well as, inactivation or deletion of tumor suppressor genes such as: *p*53, *Rb*-1, and *BRCA*1 (3). Despite these clinical correlations, at present it is not yet possible to ascribe a pivotal role for a specific genetic mutation in breast cancer aetiology. To clarify this issue, it would be desirable to follow the progressive changes until malignancy ensues in the tissue *in vivo*. However, the time-course of breast tumor evolution can be quite long, taking anywhere from 5-30 years to develop. To follow the development of carcinoma in situ to invasive carcinoma, epidemiological protocols must span periods of 10-15 years(4). This precludes the feasibility of conducting meaningful and reproducible human studies and emphasizes the need to develop appropriate experimental models of human breast cancer progression in culture.

The stroma of the mammary gland accounts for more than 80% of the resting breast volume (5). This stroma consists of fibroblasts, blood vessels, and a macromolecular network composed of glycoproteins and proteoglycans, known collectively as extracellular matrix (ECM). It has been demonstrated that this tissue microenvironment plays a fundamental role in both the control of luminal epithelial cell gene expression, and the induction and maintenance of their tissue-specific function. Studies using rodent models have revealed the importance of a reconstituted basement membrane to the morphogenesis and differentiation of mammary luminal epithelial cells (6). When placed upon a reconstituted-basement membrane gel, mammary epithelial cells undergo morphogenesis to form spheroids that exhibit many of the structural and functional characteristics of alveoli *in vivo* (7,8).

There are tissue differences between rodents and human which need to be addressed. In contrast to the mouse, human mammary epithelial cells (MECs) are not embedded directly in fat tissue. Instead, they are surrounded by a separate intralobular stroma, consisting of collagenous connective tissue and varying amounts of resident fibroblasts lying as scattered single cells. The adipocyte and epithelial compartments, in turn, are separated and surrounded by a dense interstitial stroma, which is inhabited by blood vessels (9). In addition to species differences in normal tissue architecture, the breast cancers produced in rodents are not morphologically identical, nor do they invade and metastasize the same as human breast cancers (10). Therefore, a dissection of the functional significance of these interactions in the genesis of human breast cancer will, in our opinion, benefit from the design of novel culture systems for human cells to address these questions.

Studies using a 3-dimensional basement membrane gel culture system in rodents have elucidated mammary-specific responses to ECM at the morphogenetic, cellular and molecular levels. The same rationale have now been applied to normal and neoplastic human MEC lines and primary cultures. Defined cell-ECM interactions can be exploited rapidly and accurately to distinguish normal and malignant breast cells in culture (11). Using this assay system, we demonstrated the capacity of normal human MECs to re-express their in vivo patterns of growth and differentiation. Cells from reduction mammoplasties and non-malignant cell lines including HMT-3522 and MCF10A formed polarized organotypic spheres resembling acini *in situ.* These cells also deposited an endogenous basement membrane and subsequently growth arrested after 7-10 days of culture. In contrast, primary breast carcinoma and tumorigenic breast cell lines formed large, dense and unpolarized colonies of cells and failed to deposit basement membrane or growth arrest. Subsequently, similar differences between normal and malignant primary cells were observed by Bergstresser and

Weitzman (1993) when cultured on basement membrane gels (12). Based on these differences, we hypothesized that the ability to sense the basement membrane correctly could be the function of a new class of tumor suppressor genes and their functions were presumably lost when cells became malignant (11).

One important parameter of mammary differentiation that is poorly understood is the expression of the iron binding protein lactoferrin (LTF). This evolutionarily conserved glycoprotein is a member of the transferrin gene family. Originally identified in milk, its iron binding properties are thought to endow LTF with growth promoting, iron transport and bacteriostatic properties (13,14). Unlike β–casein and whey acidic protein, significant quantities of LTF are synthesized in the normal resting mammary gland of both mice and humans. The expression of LTF mRNA and protein is often decreased or absent in premalignant and malignant human breast tissue compared to normal tissue (15).

In recent years there has been an interest in the possibility that microenvironmental regulators of epithelial function may influence the process of tumor formation (16-18). Epithelial cells affected by carcinogen exposure incur genetic lesions that alter growth, cell-cell and cell-ECM interactions and ultimately the invasion of stroma and metastatic spread (19-21). Genetic lesions that have been observed in breast epithelial cells include activation and overexpression of the dominantly acting oncogenes such as ras and myc and erb-B-2 and inactivation or loss of tumor suppressors such as RB, p53, and nm23 (22-28). However, it is now accepted that in many tissues, alterations in the activity of a specific gene such as a dominant oncogene is insufficient to explain the complexities of tumor formation. For example in the rodent mammary gland transplantation of ras and myc transformed mouse mammary epithelial cells into cleared fat pads can induce dysplastic ductal lesions that only sporadically progress to frank malignancy (29-31). Similarly in MMTV/c-myc and MMTV/v-Ha-ras transgenic mice, overexpression of these genes in mammary tissue does not always lead to tumors (32,33) suggesting that other changes are required.

Previous work have shown that altered interactions between tumor cells and their external signalling environment (stroma and ECM) are a hallmark of the neoplastic process. However, it is interesting to note that many, if not all, the changes observed in tumor progression also occur at various stages of normal breast development albeit under strict regulation and in accordance with tissue economy (34). This suggests that in cancer, not only is there potential for an altered stromal/ECM environment to influence tumor formation, but also for the tumor to respond inappropriately to normal microenvironmental cues. Thus, under normal circumstances the regulation provided by normal microenvironment,

particularly the ECM, may have a suppressive influence on tumorigenesis and progression (35-36). Of particular interest in this regard is the observation that transfection of the $\alpha 5\beta 1$ integrin receptor led to deposition of fibronectin into their matrix and reduced migration (38). These cells also showed reduced saturation density , reduced colonization of soft agar and complete suppression of tumorigenicity compared to non-transfected controls. This indicates that replacement of a lost ECM receptor in aggressive tumor cells can restore the anchorage dependence of these cells and inhibit their tumor forming potential. Metastatic suppressor effects have also been indicated for the cell adhesion molecule E-Cadherin since antibodies that block E-Cadherin function induce invasive behavior in non-transformed MDCK epithelial cells (39). Taken together, these data emphasize that while abnormal responses to microenvironment are a general characteristic of tumors, proper intercellular communication and cell-ECM interactions as mediated by cadherins, integrins and cytoskeleton may be important in suppressing the tumor phenotype.

RESULTS/EXPERIMENTAL METHODS

The objectives outlined in the original grant proposal were to characterize the molecular mechanisms whereby normal versus tumor cells interacted with their extracellular matrices (ECMs) and to determine the functional significance of this interaction thereby clarifying the role of competent ECM/cell signalling in tumorigenesis. These types of studies are anticipated to identify dominant tumor suppressor genes critical to the maintenance of the normal mammary epithelial cell phenotype. We outlined three specific goals in our original proposal to achieve these objectives.

Specific goal 1 concerned defining which ECM ligands were critical to breast epithelial function by challenging isolated cells with purified ECM components. Towards this goal we have conducted a number of experiments using both mouse and human MECs and manipulated the cell shape and extracellular environment to ascertain their effects on cell differentiation. We used lactoferrin as a new marker for MEC differentiation because it is expressed in vivo in normal mouse and human mammary epithelium and is down regulated in a significant proportion of human breast tumors. Initial studies were conducted using the murine mammary epithelial cell line, CID-9 and its clonal derivative SCp2. These studies were then extended to freshly isolated human mammary epithelial organoids isolated from reduction mammoplasty tissue and early passage cells derived from the organoids.

These studies have resulted in the manuscript "Extracellular matrix regulation of lactoferrin expression in mouse and human mammary epithelial cells is mediated by changes in cell shape" which is being submitted for publication (see attached and appendix). The results are summarized below.

Lactoferrin expression is regulated by cell shape and modulated by extracellular matrix in mouse & human mammary epithelia
Mouse mammary epithelial studies:

A) Basement membrane components induce lactoferrin gene expression in cultured mouse mammary epithelial cells

Neither the original murine mammary epithelial cell line, CID-9, nor its clonal derivative SCp2 showed detectable levels of lactoferrin mRNA when cultured under standard conditions on plastic. Tissue specific gene expression in these cell lines has previously been shown to depend on lactogenic hormones and contact with reconstituted basement membrane (7,8,40,41). In order to determine the effect of basement membrane on the induction of lactoferrin expression in mammary epithelium, CID-9 and SCp2 cells were cultured in the presence or absence of complete reconstituted basement membrane (EHS-matrix) or purified laminin, a major basement membrane component. Cultures were examined for lactoferrin expression by northern analysis. Lactoferrin (LTF) mRNA expression could be induced in CID-9 and SCp2 cells by plating the cells on EHS matrix or by adding small amounts of EHS or purified laminin to the culture medium. The expression of LTF was independent of the mammotrophic hormone prolactin. In contrast, when these cells were plated at similar density on plastic in either 5% serum containing or serum-free medium, LTF expression was not observed. After prolonged growth of SCp2 cells in 5% serum containing medium, however, LTF was expressed without addition of exogenous basement membrane, suggesting that cell shape changes due to crowding, increased cell cohesion, or endogenous ECM deposition could recapitulate the conditions for LTF induction provided by basement membrane.

B) Cell-cell interaction promotes lactoferrin expression in the absence of exogenous extracellular matrix

Since high density cultures of SCp2 cells express LTF mRNA when stimulated by 5% fetal bovine serum, we examined the role of cell-cell interaction in this inductive process by culturing SCp2 cells on polyHEMA coated substrata where they were forced to cluster in the absence of serum

or exogenous ECM. In order to distinguish between decreased cell-substratum adhesion and increased cell-cell cohesion as the driving force behind the induction of LTF expression, we lowered the levels of extracellular Ca^{2+} so that calcium dependent intercellular interactions would be decreased. Lowering the extracellular calcium concentration to 50 μM in non-adherent polyHEMA cultures decreased the number of large cell clusters and increased the proportion of single cells. In adherent cultures, lowering the extracellular calcium concentration to 50 μM reduced the extent of cell spreading and abolished cell-cell interaction. In both suspended and attached cultures, lowered calcium levels were accompanied by increased LTF expression. Thus, decreased cellular adhesion, rather than increased cell-cell cohesion appears to be sufficient for LTF expression.

C) Cell rounding in the absence of cell-ECM or cell-cell interaction is sufficient for lactoferrin expression

In order to further investigate the possibility that cell rounding alone could be sufficient to trigger LTF expression, we asked whether lactoferrin expression could be induced in single, suspended, mammary epithelial cells in the absence of either ECM or intercellular contact. LTF expression was analyzed immunochemically in suspended SCp2 cells cultured on polyHEMA with low (50 μM) Ca^{2+} and in adherent SCp2 cells on plastic with normal (2mM) Ca^{2+}. LTF protein was expressed at high-levels in single cells harvested from polyHEMA cultures but not in cells cultured on plastic. These data suggest a change in cellular cytoskeleton is sufficient to induce LTF expression. Under physiologically relevant conditions i.e., in the presence of basement membrane, an appropriate cell shape can be maintained. If the shape of the cell is transfigured by increased substratum adhesion and cell spreading then lactoferrin expression is switched off.

Human mammary epithelial studies:
A) Lactoferrin expression is induced in human mammary epithelial cells cultured on basement membrane or in suspension

Lactoferrin is abundantly expressed in normal human breast epithelium in vivo and marked changes in expression are a common occurrence in pre-malignant and malignant breast tissue suggesting that lactoferrin may be an important marker of breast function that is deregulated in malignancy. In order to investigate the signalling requirements for LTF expression in human MECs and establish a role for basement membrane in this process, we cultured organoids isolated from

surgically removed reduction mammoplasty tissue and early passage cells derived from the organoids on EHS-matrix and polyHEMA. LTF mRNA is abundantly expressed in organoids, but is down regulated in 4th or 5th passage human MEC's cultured on plastic. In contrast, under similar conditions, 4th passage human MEC cells plated on EHS-matrix in the presence or absence of EGF show high-level expression of LTF. Lactoferrin is also expressed in 5th passage human MEC cells that are forced to cluster by culture on polyHEMA coated dishes. Since cell clustering induced by polyHEMA has been shown to induce endogenous basement membrane synthesis in mouse mammary epithelial cells, we investigated whether the induction of lactoferrin in polyHEMA cultures was contingent on the induction of an endogenous basement membrane. We previously reported the impairment of β1 integrin ECM signalling (intact cell/ECM interactions) prevented competent β-casein synthesis in mouse MECs (8), thus we cultured organoids and human MEC's on EHS-matrix and polyHEMA in the presence or absence of an inhibitory anti-β1 integrin antibody. Anti-human β1 integrin antibodies blocked the expression of LTF mRNA in organoids and early passage HMEC's on both EHS-matrix and polyHEMA. In contrast, LTF expression was maintained in the presence of non-immune IgG. These data suggest that ECM can modulate cellular shape necessary for the expression of lactoferrin and that this interaction is dependent on ECM-β1-containing integrins. Further studies are underway to clarify which ECM components are sufficient for lactoferrin induction. Initial studies will involve manipulating laminin and entactin levels using either the two dimensional matrix overlay assay or the polyHEMA system. Future studies are envisioned using dominant negative mutant β1 integrin constructs to clarify which integrin signalling components are critical to this LTF induction pathway.

Specific goal 2 concerned identification of the specific integrins (ECM receptors) that transduce the ECM signals to the cells, by examining their expression in normal and tumor cells and elucidating their functional role by disrupting the signalling pathways. Towards this goal we have examined the profiles of integrin expression in a number of tumorigenic human mammary epithelial cell lines and compared this with those expressed by a phenotypically normal human mammary epithelial cell line. In addition, we have conducted a number of integrin blocking studies. The results from these studies have been recently published as "Cellular growth and survival are mediated by β1 integrins in normal breast epithelium but not in some breast carcinoma" (see attached and appendix). These results are summarized below.

Cellular growth and survival are mediated by β1 integrins in normal breast epithelium but not in some breast carcinoma

A) The profile of integrin subunits expressed by normal and tumorigenic human breast epithelial cells in vivo is recapitulated in three-dimensional culture

To determine whether normal and tumor cells, cultured within reconstituted basement membrane, express a profile of integrins similar to that shown in vivo, normal HMT-3522 cells and tumorigenic HMT-3909/S13, MCF-7 subline 9 (MCF-7/9), and MDA-MB-435 breast carcinoma cells were cultured in EHS matrix for 12 days. Cultures were examined for the expression of integrin subunits by immunocytochemistry. The normal and tumorigenic cell lines were found to broadly recapitulate the pattern of integrin subunit expression and localization predicted from published in vivo data. In normal HMT-3522 cells, the strongest staining was seen for the α3 and β1 subunits, followed by α6 and β4 subunits. In contrast, all the tumor cells showed loss, disordered expression, or downregulation of these integrin subunits.

B) Formation of acinar structures within EHS is integrin dependent

To determine which of the various integrins expressed by human breast epithelial cells are functionally relevant to the formation of acinar structures, specific inhibitory anti-integrin antibodies were used to interfere with this process in reconstituted basement membrane culture. Normal HMT-3522 cells cultured in EHS without antibodies, or with 10 μg/ml or 100 μg/ml of non-immune mouse or rat IgG, formed well-organized acinar structures at similar frequencies. In contrast, inhibitory anti-β1 integrin subunit antibodies, at similar concentrations, severely impaired the formation of spheres by HMT-3522 cells relative to control cultures. These effects were observed with two different anti-β1 antibodies and were dose dependent. Similar results were obtained at day 6 and day 12 of culture. These data suggest that sphere formation by normal human mammary epithelial cells in response to EHS is dependent on intact β1 integrin ECM interactions.

C) The inhibition of acinar morphogenesis by anti-integrin antibodies is associated with an inhibition of cell growth

To determine whether the inhibitory antibodies interfered with acinar formation by blocking cellular growth, the thymidine-labeling indices (TLIs) of normal HMT-3522 in EHS were determined at day 2 and

day 6 of culture. In the absence of inhibitory anti-β1 antibodies the TLIs were approximately 60% at day 2 but fell to 3% by day 6 as the cells formed differentiated acini. In contrast, in the presence of inhibitory anti-β1 antibodies the cells remained suspended as single cells, and the TLIs were low at day 2 and remained low throughout the experiment. These data suggest that an initial phase of cell growth is a requirement for acinar formation in three-dimensional culture. Thus, acinar formation appears to be a two-step process involving a β1-integrin-dependent cellular growth phase, followed by a phase of cell polarization to form the final organized structures.

D) Interruption of normal mammary cell-basement membrane interactions induces apoptosis

Prevention of appropriate cell-ECM contact by use of non-adhesive (polyHEMA) coated substrata, ECM fragments or RGD peptides can inhibit cell growth and differentiation in anchorage-dependent cells (42,43) and trigger programmed cell death or apoptosis (44). We have shown that inhibition of mammary cell attachment to basement membrane by ligation of β1-integrins blocks cellular growth and acinar formation. In the mouse cells, we had found tht long-term homeostasis is dependent on an intact ECM. We therefore asked whether the inhibition of mammary cell-BM interaction induces apoptosis. In the absence of anti-β1 antibodies, HMT-3522 cells formed acinar structures as described above. Apoptotic nuclei were detected infrequently (0.74%) at day 2, whereas at day 6, 6.3% of single cells not incorporated into acini and 2.5% of individual cells with acini were stained with the ApopTag reagent. In contrast, in the presence of anti-β1 antibodies, 21% of the cells contained nuclei stained by ApopTag reagent at day 2 and at day 6, 60% of the nuclei were labelled. These data confirmed the finding in the mouse model that β1 integrins transmit signals from ECM that are required for cell survival.

E) Inhibitory anti-integrin subunit antibodies do not block colony formation by breast carcinoma cells in reconstituted basement membrane

To determine whether the formation of colonies by breast carcinoma cells in reconstituted basement membrane reflects a failure of the cells to sense BM correctly, the effects of inhibitory anti-integrin antibodies were tested on HMT-3909/S13, MDA-MB-435 and MCF-7/9 cells. We found that inhibitory anti-β1 antibodies did not inhibit the capacity of tumor cells to make colonies within EHS at the concentration that inhibited acinar formation in the nontumorigenic normal HMT-3522 cell line. We then asked whether inhibitory anti-integrin antibodies could influence the

growth properties of breast carcinoma cells or induce apoptosis in three-dimensional culture. HMT-3903/S13, MDA-MB-435 and MCF-7/9 cells were cultured in Matrigel for 6 days in the presence or absence of anti-β1 antibodies. Control culture (no antibodies) of HMT-3909/S13 and MDA-MB-435 carcinoma cells showed moderate TLIs of approximately 10% and MCF-7/9 demonstrated a TLIs of 25%. No inhibition of cellular growth was observed in any of these tumorigenic cell lines in the presence of inhibitory anti-β1 antibodies. These data demonstrate that despite competent β1 integrin expression by these carcinoma cell lines, tumor cells have escaped the restraining influences of ECM for both growth and apoptosis.

F) The mechanism whereby tumorigenic MEC's circumvents signalling through the β1 integrin pathway

Our lactoferrin studies have emphasized the importance of competent β1 integrin ECM signalling in the maintenance of the differentiated phenotype in nontumorigenic human mammary epithelial organoid cultures (see goal 1). Consistent with these results we observed a comparable integrin profile in our nontumorigenic HMT-3522 MEC line when cells were grown in a reconstituted basement membrane to that reported in vivo in the literature. Interruption of this signalling pathway was shown to lead to inhibition of morphogenesis and induction of apoptosis. In contrast we have observed a uniform loss of and/or disorganized expression of the β1 integrin subunit in a number of tumorigenic mammary epithelial cell lines. In addition we have consistently observed a refractoriness to either colony formation or apoptosis induction by blockage of this β1 signalling pathway in all of our tumor cell lines. Therefore we decided to focus on the mechanism whereby tumorigenic mammary epithelial cells are able to circumvent signalling through this pathway in an effort to determine if this comprises a critical step in the tumorigenic process. To achieve this we have gained access to the tumorigenic counterpart of the phenotypically normal HMT-3522 MEC currently used as normal cell population. These cells have acquired since Dr. Howlett joined Berlex, a biotech company, and the experiments have been conducted by Drs. Valerie Weaver and Huei-Mei Chen whom we are now training to lead this project. Similar to our previous studies on both primary cultures and immortalized cell lines, the nontumorigenic HMT-3522 cell line forms organized spheroids, deposits an intact basement membrane and undergoes growth arrest 6-8 day after culturing in a reconstituted basement membrane while its tumorigenic counterpart forms disorganized spheroids and fails to deposit a basement membrane or growth arrest (Fig. 1). Initial descriptive studies have confirmed similar integrin profiles are expressed by both the

nontumorigenic and tumorigenic cell lines although the organization is severely compromised in the tumorigenic line (unpublished data). Interestingly, similar to our previous studies, blockage of β1 integrin signalling in the tumorigenic cells failed to prevent colony formation or induce apoptosis (45). We are currently delineating the basis for this refractoriness. Future studies will involve overexpression of functional β1 integrin constructs into the tumor cells in an attempt to correct this defect or the transfection of a dominant/negative β1 integrin mutant into the normal HMT-3522 cells to determine if this will eventually give rise to a tumorigenic clone. Using this novel paired cell population we will be able to manipulate signalling through the β1 integrin receptors against a similar genetic background.

Specific goal 3 involved evaluating the function of known suppressor genes such as nm23, p53 and RB and ascertaining their roles in the regulation of breast cell/ECM interaction by transfecting these genes into breast tumor cells and assessing the consequence of expression on tumor cell growth and differentiation. We have completed a series of studies concerning the role of the nm23 gene and its consequent effects on the tumor phenotype. This work has resulted in a publication "A novel function for the nm23-H1 gene: overexpression in human breast carcinoma cells leads to the formation of basement membrane and growth arrest" (see attached and appendix). The results are summarized below.

Overexpression of nm23-H1 in human breast carcinoma cells leads to normalization of cell morphology, formation of basement membrane, production and apical secretion of sialomucins and growth arrest

To determine whether or not nm23-H1 gene expression had any functional consequences in the 3-dimensional assay, a metastatic MDA-MB-435 breast carcinoma clonal cell lines were transfected with pCMVBamneo vectors (C-100 and C-103) or the same vector containing the full length nm23-H1 cDNA (H1-170 and H1-177). Cell were cultured within an EHS matrix for 12 days. Cultures were examined for Nm23 protein expression by immunohistochemistry and for the presence of acinus-like structures by microscopy. Both the parent MDA-MB-435 cell line as well as the control clones expressed little Nm23 protein and produced large disorganized colonies. In contrast, the H1-177 transfectant expressed significant Nm23 protein and produced small spheres with occasional lumens or remained as single cells. The morphology of the H1-177 cells and Nm23 protein-positive H1-170 cells differed from that of the previously characterized HMT-3522 cells (11) only in the proportion of spheres with a central

lumen. Less than 1% of the Nm23 protein -positive spheres contained a lumen.

In addition to morphological evidence of differentiation, cultures of normal HMT-3522 cells within an EHS matrix induced the expression of basement membrane proteins and their deposition to the outside of the acinus-like spheres (11). Analysis of six breast carcinoma cell lines and two primary carcinoma cultures failed to show basement membrane deposition. Immunohistochemical staining of type IV collagen and laminin was conducted on parental MDA-MB-435 cells, control transfectants, and nm23-H1 gene transfectants cultured within an EHS matrix . By day 6 of culture, 87.8% of Nm23 protein-positive transfectants deposited type IV collagen; this percentage increased to 97.1% by day 12 of culture. These latter data compared closely with the normal HMT-3522 cultures, where almost all spheres deposited a basement membrane by day 12 of culture. Basement membrane proteins were localized to the outside of the acinus-like spheres. In contrast, none of the parental cells, control transfectants, or Nm23 protein-negative H1-170 transfectants expressed type IV collagen or laminin.

There is a difference in both the morphology and size of colonies produced by control and nm23-H1 gene transfectants when cultured within an EHS matrix. The parent line and control transfectants produced colonies ranging from 16-26 cells per colony. H1-177 cells produced colonies containing an average of 9.6 cells, which compared closely with the 8.0 cells per sphere exhibited by normal HMT-3522 cells. Analysis of H1-170 cells further strengthened this trend; Nm23 protein-negative colonies contained a mean of 27 cells, while Nm23 protein-positive colonies contained a mean of 8.2 cells.

The relationship of biosynthetic and growth inhibition aspects of breast cell differentiation have been evaluated in the H1-177 cell line. Deposition of basement membrane was prevalent among colonies by day 6 of culture (87.8%) and virtually homogeneous by day 12 of culture (97.1%). In contrast, the percentage of thymidine labeled cells remained high at day 6 of culture (92%) but was reduced to 29.9% by day 12. The percentage of spheres that were basement membrane positive-[^{3}H]thymidine negative rose from 7.6% on day 6 of culture to 70.1% on day 12. A concurrent decrease in the percentage of basement membrane-positive-[^{3}H] thymidine-positive sphere was observed, from 80.2% on day 6 to 27.0% on day 12. All the remaining basement membrane-negative spheres were [^{3}H]thymidine positive on day 12 of culture. The data suggest the hypothesis that basement membrane synthesis and secretion, an early event in this system, may signal an inhibition of cell growth. Taken

together, the data provide evidence of an antiproliferative effect of nm23-H1 gene expression in breast epithelial cells.

Future studies are planned to examine the role of the p53 and RB genes in the regulation of breast epithelial cell/ECM interactions using the MDA-MB468 and BT549 breast carcinoma cell lines transfected with either RB or p53 as described in our previous grant proposal. Expression of single copy of these genes reduced the ability of the cells to grow in soft agar and their tumorigenicity in nude mice. Thus similar to the nm23 gene we anticipate RB and p53 transfected cells will demonstrate effects on cell morphology when cultured in a reconstituted basement membrane. Since reconstituted basement membrane cultures require serum free adapted cells, we have begun adapting these cell lines to serum free conditions.

CONCLUSION

In this report we have summarized our findings during the period of July 1, 1994 - September 30, 1995. These results have been put together in three published papers and one submitted manuscript (see appendix). We believe this is tremendous progress. The most recent data on regulation of LTF expression revealed the differential response of three milk proteins (LTF, β-casein and whey acidic protein) to ECM and hormonal signals. Although production of all three proteins can be induced by a complete basement membrane, only LTF mRNA is increased at high cell densities in the absence of exogenous ECM. Moreover, LTF expression was insensitive to prolactin stimulation whereas β-casein expression was absolutely dependent on this lactogenic hormone. Interestingly, LTF expression can be changed simply by manipulating the culture medium compositions, i.e, calcium level or replacing plastic with poly(HEMA). Our data suggest that cell rounding by decreasing cell adhesion and changes in the cytoskeleton could trigger LTF expression and provide evidence for mechanochemical signalling pathway in regulating milk protein production.

In vivo, change of ECM components of the mammary gland during different stages of growth and differentiation may provide different physical constraints on the shapes of responsive cells. These physical constraints may, in turn, dictate structural configurations of protein complexes on cell surfaces. Signals provided by ECM are known to be transduced by integrins, which are α/β heterodimeric transmembrane receptors (46). Ligation of integrins can initiate intracellular biochemical events, including flux in calcium and cAMP levels, activation of the Na^+/K^+ antiporter, and increase in tyrosine phosphorylation that propagate the ECM signals within the cell (47,48). More recently, integrins have been found to operate as mechanochemical receptors which transmit mechanical signals across the cell membrane to the cytoskeleton (49).

Experiments are currently in progress in our laboratory to determine whether specific integrins are involved in cell shape dependence of LTF gene expression, and how changes in cell shape are translated into changes in LTF mRNA abundance within responsive cells.

Human MECs in vivo express several integrins that recognize laminin. The expression of these integrins has been found altered in the majority of human breast carcinomas studied and the severity of receptor losses or downregulation correlates with tumor grade(50-52). This suggests that a dysfunction of integrin signalling may be an important parameter in breast tumorigenesis. Our data showed that the changes in integrin levels were most severe in the metastatic MDA-MB-435 cells, suggesting a correlation between loss of regulation of integrin expression and aggressive tumor behavior consistent with the in vivo survey data. All these results validate previous reconstituted basement membrane assay, provide new markers of differentiation to distinguish normal and malignant breast epithelial cells in our 3-dimensional assay, and provide a basis for evaluating the functional role of integrins in mediating signals from ECM for mammary-specific differentiation in culture.

Through the use of specific inhibitory anti-integrin antibodies, we have shown that the morphogenesis of acinar structures by normal breast epithelial cells is dependent on integrins. Furthermore, specificity of integrin signaling is dependent on the nature of the ECM presented to the cells. In addition, we have observed a growth-inhibitory effect associated with the anti-integrin-mediated block to acinar morphogenesis in EHS matrix and collagen I. Overall, our data support the hypothesis that if integrin-mediated contact with ECM is disturbed, the cells can neither grow nor differentiate and are unable to survive. On the other hand, the antibodies did not inhibit carcinoma cell growth or induce apoptosis suggesting that tumor colony formation occurs independently of the integrin signalling utilized by normal cells. Taken together, our data demonstrate that normal breast epithelial cells form acinus-like structures from single cells by a process that involves at least two distinct phases:(1) growth; and (2) conversion of the cell group into differentiated structures. Integrins probably are important in both phases of normal behaviors. Breast carcinoma cells form disordered colonies independently of β1 integrin; cellular growth is not inhibited and apoptosis is not induced by antibody blockade. Thus, defects in cellular responses to microenvironment, including ECM, may be an important parameter of malignancy in addition to other established genetic lesions. The mechanisms by which breast carcinoma cells escape integrin-mediated regulation of growth and apoptosis remain to be determined and elucidation of this process will be a valuable step toward further understanding the nature of human breast malignancy.

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APPENDIX (publications and manuscripts from research progress during July 1, 1994 - September 30, 1995):

1. Howlett AR, Close MJ, Roskelley CD, Desprez PY, Bailey N, Teng CT, Stampfer MR, Bissell MJ and Yaswen P (1995) Extracellular matrix regulation of lactoferrin expression in mouse and human mammary epithelial cells is mediated by changes in cell shape. Manuscript submitted.

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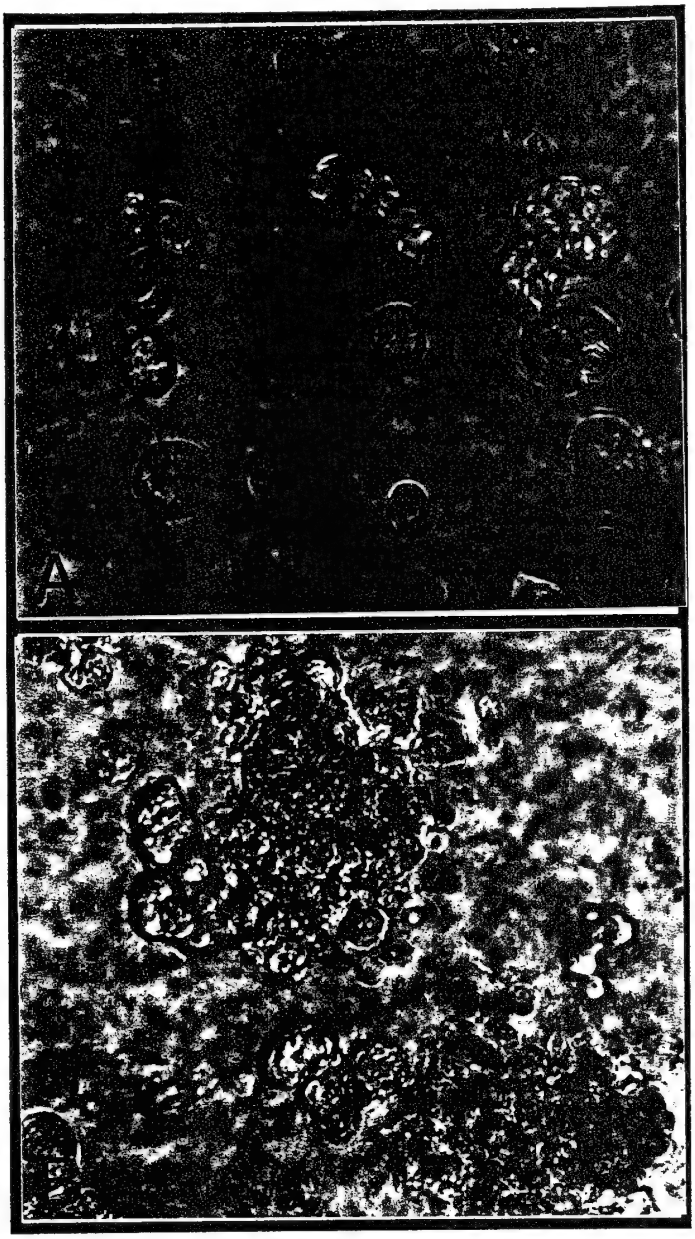
FIGURE LEGEND

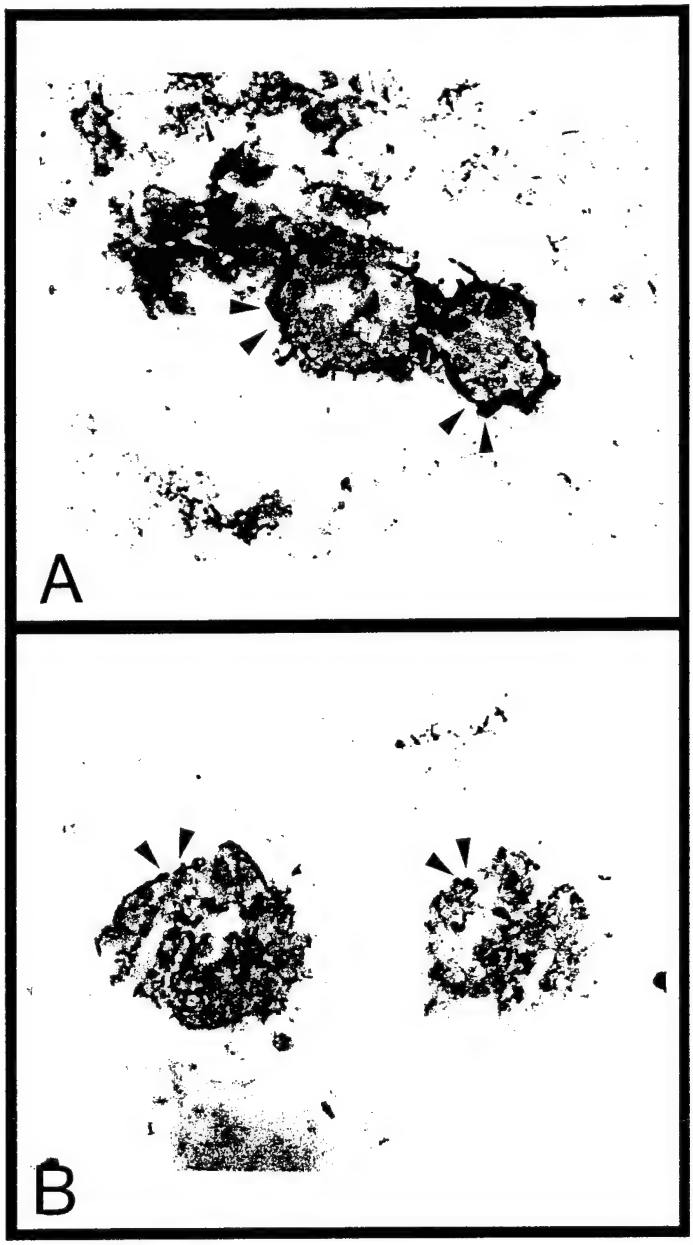
Fig. 1. a) Morphological examination of HMT-3522 cell passages cultured in 3- dimensional basement membrane (EHS) for one month. Phase contrast micrographs of normal (A) S-1 passage 50 cells and malignant (B) T-4 passage 20 cells. Normal S-1 passage 50 cells formed normal, organized spheroids while malignant T-4 passage 20 cells formed disorganized and large spheroids. **b)** Bright field micrographic examination of normal and malignant breast epithelial cells cultured in basement membrane gels for 4 weeks and examined for the presence of deposited endogenous basement membrane. Bright field micrograph of normal (A) and malignant breast epithelial cells (B). 5 micron cryostat sections of basement membrane gels cultured for 4 weeks and immunostained for collagen IV. Only normal HMT-3522 early passage S-1 50 cells deposited a basement membrane. **c)** Kinetics of cell growth by normal S-1 HMT-3522 and malignant T-4 20 HMT-3522 cells grown in a reconstituted basement membrane (EHS) for up to 10 days. Kinetics of cell grown for S-1 50 HMT-3522 normal and T-4 20 HMT-3522 malignant cells when grown within a reconstituted basement membrane for up to 10 days. Cells were seeded at equal densities in EHS and assayed for cell number at 2, 4, 6, and 10 days after seeding. Normal HMT-3522 cells ceased growing and underwent morphogenesis by 6 days after culture in EHS while malignant cells continued growing.

a) MORPHOLOGICAL EXAMINATION OF HMT 3522 CELL PASSAGES CULTURED IN 3 DIMENSIONAL BASEMENT MEMBRANE (EHS) FOR 1 MONTH



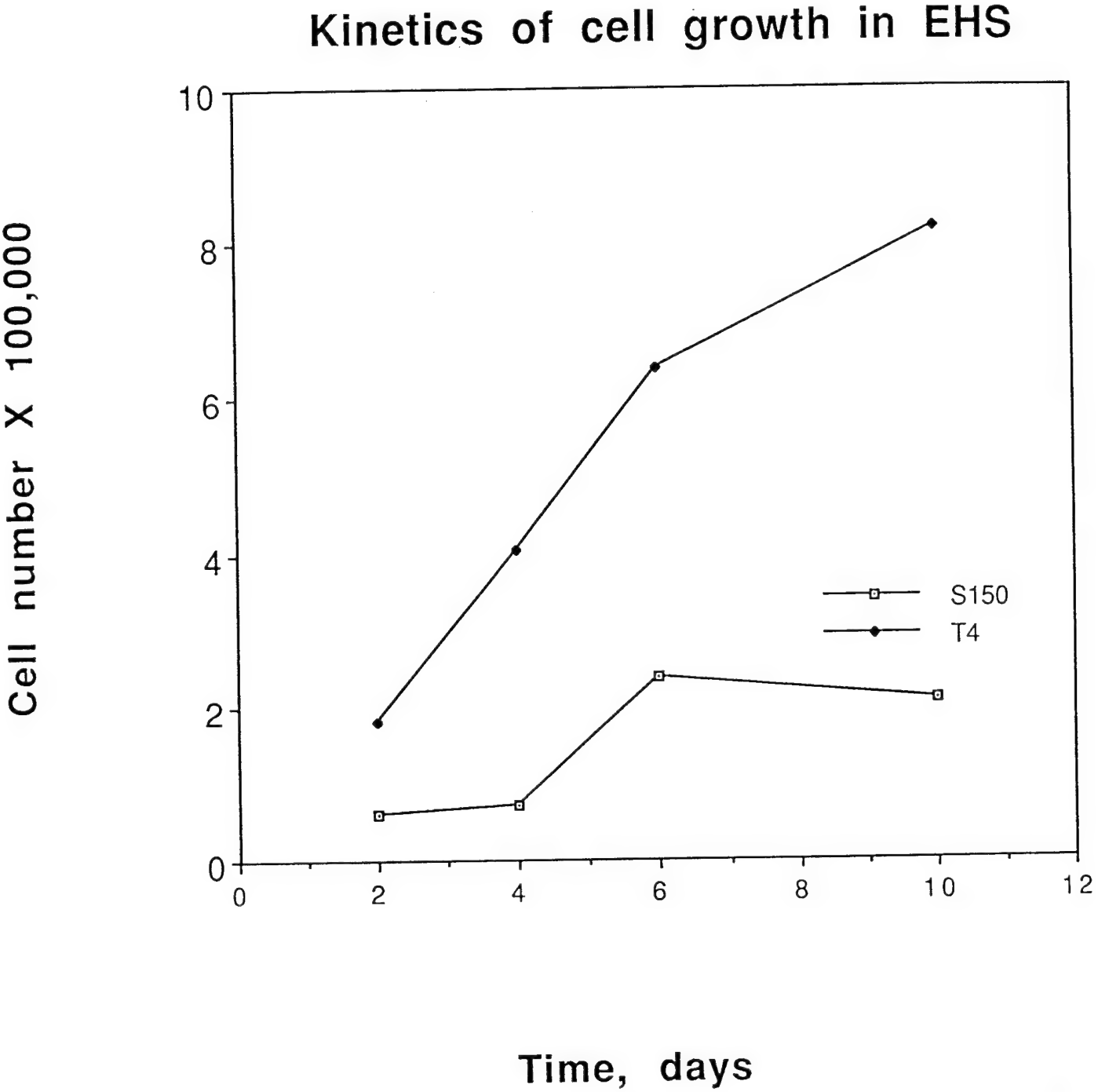
Phase contrast micrographs of normal (A) S-1 passage 50 cells and malignant (B) T-4 passage 20 cells. Normal S-1 passage 50 cells formed normal, organized spheroids while malignant T-4 passage 20 cells formed disorganized, large spheroids.

b) BRIGHT FIELD MICROGRAPHIC EXAMINATION OF NORMAL AND MALIGNANT BREAST EPITHELIAL CELLS CULTURED IN BASEMENT MEMBRANE GELS FOR 4 WEEKS AND EXAMINED FOR THE PRESENCE OF DEPOSITED ENDOGENOUS BASEMENT MEMBRANE



Bright field micrograph of normal (A) and malignant breast epithelial cells (B). 5 micron cryostat sections of basement membrane gels cultured for 4 weeks and immunostained for collagen IV. Only normal HMT-3522 early passage S-1 50 cells deposited a basement membrane.

c) KINETICS OF CELL GROWTH BY NORMAL S-1 HMT 3522 AND MALIGNANT T-4 20 HMT 3522 CELLS GROWN IN A RECONSTITUTED BASEMENT MEMBRANE (EHS) FOR UP TO 10 DAYS.



KInetics of cell growth for S-1 50 HMT 3522 normal and T-4 20 HMT 3522 malignant cells when grown within a reconstituted basement membrane for up to 10 days. Cells were seeded at equal densities in EHS and assayed for cell number at 2, 4, 6 and 10 days after seeding. Normal HMT 3522 cells ceased growing and underwent morphogenesis by 6 days after culture in EHS while malignant cells continued growing.

EXTRACELLULAR MATRIX REGULATION OF LACTOFERRIN EXPRESSION IN MOUSE AND HUMAN MAMMARY EPITHELIAL CELLS IS MEDIATED BY CHANGES IN CELL SHAPE

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Short title: Regulation of lactoferrin in mammary epithelium

Key words: Mammary epithelium, extracellular matrix, basement membrane, lactoferrin, cell shape.

SUMMARY

Lactoferrin is a secreted iron binding protein which is abundantly expressed by mammary epithelial cells at many stages of functional development, including non-pregnant, non-lactating stages, and which shows marked changes in expression in premalignant and malignant breast tissue. In adherent cultures of mouse or human mammary epithelial cells grown under standard conditions on plastic, expression of lactoferrin is suppressed. However, we have found that lactoferrin can be reexpressed in a murine mammary epithelial cell line or early passage cultures of normal human mammary epithelial cells when these cells are exposed to a gel of exogenous basement membrane components. We have proceeded to use this differentiation marker to identify factors which regulate the ability of mammary epithelial cells to sense and respond to their local microenvironment. We have found that lactoferrin expression, unlike that of β-casein, does not require the presence of specific exogenously added extracellular matrix components or mammatrophic hormones. Culture conditions (superconfluence, cell suspension) which increased cell-cell cohesion while minimizing cell adherence and spreading on rigid, impermeable surfaces were sufficient to restore expression of this differentiated marker. These data suggested that extracellular matrix may influence lactoferrin expression either by altering cell-cell interactions or by providing mechanochemical signals that regulate cell shape. Evidence for the latter came from analysis of single rounded cells maintained in low calcium medium in either adherent or suspension cultures. In either case, lactoferrin expression was observed in the absence of obvious cell-cell interactions. Thus, the influence of extracellular matrix on the expression of lactoferrin most likely occurs primarily by the conveyance of mechanochemical signals through structural constraints.

INTRODUCTION

The mechanisms by which the expression of tissue-specific genes are induced and maintained is central to the understanding of normal tissue development and differentiation. Cells can transduce microenvironmental cues that regulate cellular function from a variety of sources, including soluble hormones and cytokines, cell-cell interactions, and interactions with extracellular matrix (ECM; for review see Stoker et al., 1990; Watt et al., 1986; Howlett and Bissell, 1993). The ECM itself influences cellular function by a variety of mechanisms including classical ligand-receptor interaction, promotion of cell-cell cohesion, and modulation of cell shape (Bissell, 1981; Blau and Baltimore, 1991; Adams and Watt, 1993; Juliano and Haskill, 1993; Lin and Bissell, 1993; Frisch and Francis, 1994).

A well characterized model which utilizes murine mammary epithelial cells from pregnant or lactating tissues has shown that ECM, in conjunction with specific hormones, induces the formation of complex, highly polarized three-dimensional structures that can vectorially secrete milk specific products (Li et al., 1987; Barcellos-Hoff et al., 1989; Streuli, 1993). Recent evidence obtained using this system has revealed that appropriate cell configuration is a prerequisite for the expression of both ß-casein and whey acidic protein (WAP) in response to ECM containing basement membrane. Transcription and synthesis of ß-casein, one of the major products of functionally differentiated murine mammary epithelial cells, is maintained by contact with basement membrane, specifically by laminin binding to ß1 integrins (Roskelley et al., 1994; Streuli et al., 1991; Streuli et al., 1995). In addition, cell rounding in response to basement membrane induces a physical signal that is required for β-casein expression (Roskelley et al., 1994). In contrast, the expression of WAP, while dependent on basement membrane, requires cell-cell contact and formation of a complete alveolar structure (Chen and Bissell, 1989; Lin and Bissell, 1993). Increased cell contact during alveolar morphogenesis is required to overcome negative regulatory effects of TGF- α produced by isolated cells (Lin et al., in press). Taken together, these data suggest that ECM regulates mammary epithelial cell function through multiple signalling pathways that include modulation of cell shape.

One important parameter of mammary differentiation that is poorly understood is the expression of the iron binding protein lactoferrin (LTF). This evolutionarily conserved glycoprotein is a member of the transferrin gene family. Originally identified in milk, its iron binding properties are thought to endow LTF with growth promoting, iron transport and bacteriostatic properties (Crichton, 1990; Kijlstra, 1991). Unlike β-casein and WAP, significant quantities of LTF are synthesized in the normal resting mammary gland of both mice and humans. The expression of LTF mRNA and protein is often decreased or absent in premalignant and malignant human breast tissue compared to normal tissue (Campbell et al., 1992).

In order to evaluate the role of ECM in the regulation of LTF gene expression in mammary epithelial cells we have utilized the functional murine mammary epithelial cell lines, CID-9 (Schmidhauser et al., 1990) and SCp2 (Desprez et al., 1993), both derived from mammary tissue of mice in the middle of pregnancy (Danielson et al., 1984), as well as early passage human mammary epithelial cell (HMEC) strains, derived from reduction mammoplasty tissue (Stampfer and Yaswen, 1992). We report that cell-ECM interactions induce LTF gene expression in functional mammary epithelial cells, but that unlike β-casein, a specific ECM signal is not required. These inductive effects occur by modulation of cell shape and the transduction of a mechanochemical signal.

MATERIALS AND METHODS

Substrata

EHS matrix was prepared from EHS ascites tumors passaged in C57BL mice and used as a substratum as described (Barcellos-Hoff et al., 1989; Streuli et al., 1991; Blaschke et al., 1994). In some experiments, commercially prepared EHS-matrix was used (Matrigel, Collaborative Research, Bedford, MA). Purified laminin, poly 2-hydroxy-ethylmethacrylate (PolyHEMA) and poly-L-lysine were obtained from Sigma Chemical Co. (St. Louis, MO.). Laminin was added to the cells as an ECM overlay at 50 μg/ml as described (Streuli et al 1994). PolyHEMA was dissolved at 12 mg/ml in 95% ethanol and tissue culture plates were coated at 0.8mg /cm^2 and air

dried as described (Folkman and Moscona, 1978). Glass coversips were coated with 50 mg/ml poly-L-lysine (4 x 10^5 dalton size) dissolved in water and allowed to air dry.

Cell Culture

Mouse mammary epithelial cells of the heterogenous cell line CID-9 and clonal derivative, SCp2, were maintained in culture as previously described (Schmidhauser et al., 1992; Desprez et al., 1993). The mouse cells were plated at approximately 3 X 10^4 cells /cm^2, and were routinely maintained in DME/F12 medium supplemented with 5% fetal calf serum and insulin (5μg/ml; growth medium). For differentiation assays, cells were cultured in serum free medium with various combinations of lactogenic hormones (insulin, 5μg/ml; hydrocortisone, 1μg/ml; prolactin, 3μg/ml; differentiation medium), depending on the experiment.

Human mammary epithelial cells were prepared from reduction mammoplasty tissues. The tissue was mechanically and enzymatically dissagregated to yield epithelial organoids which were subsequently stored frozen. Monolayer cultures were established from organoids in MM media as previously described (Stampfer, 1985; Taylor-Papadimitriou and Stampfer, 1992). Normal HMEC cultures were used for experiments within the first five passages.

Culture in reduced calcium medium

Calcium chloride was added to calcium-free Minimal Essential Medium (S-MEM, Joklik modified; Life Technologies) to give final concentrations of 2mM or 50μM. SCp2 cultures were trypsinized and rinsed in calcium-free medium with 5% Chelex (BioRad) treated fetal calf serum. The cells were resuspended at a density of 4 x 10^6 cells/ml in differentiation media containing either 2mM or 50μM $CaCl_2$ and either insulin (5μg/ml) alone or insulin plus hydrocortisone (1μg/ml). Cells were seeded at 1-2x 10^6 cells per 100mm plastic dish and 2-4 x 10^6 cells per 100mm polyHEMA coated dish. SCp2 cells do not grow on polyHEMA coated dishes (data not shown), and were therefore plated at higher density to compensate for the increase in cell density on plastic during the course of the experiment.

Immunocytochemistry

Single cells were harvested from suspension culture and immobilized on poly-L-lysine coated

coverslips prior to fixation. Aggregated cells in suspension were harvested by centrifugation. Cells from suspension or adherent cultures were fixed in 2% paraformaldehyde at ambient temperature for 20 mins. Aggregated cells from suspension cultures were embedded in sucrose and frozen in Tissue-Tek OCT compound (Miles Laboratories). 5μm frozen sections were then prepared on coverslips. Cells were immunostained with polyclonal anti-mouse LTF antibodies as described (Harlow and Lane, 1988; Streuli et al., 1991). The anti-LTF antibody did not cross react with purified transferrin on Western blots (data not shown). Sections were incubated with primary antibodies for 60 mins followed by biotinylated secondary antibodies (30 mins) and Texas red conjugated streptavidin (30 mins). Control sections were stained with second antibodies only.

mRNA Analysis

Attached cultures were lysed directly in buffered guanidine thiocyanate solution, while suspension cultures were centrifuged prior to lysis in the same solution. RNA was purified and northern blots prepared exactly as described (Yaswen et al., 1992). The blots were hybridized to the following ^{32}P labelled cDNA probes: a) the 2.2 Kb mouse LTF EcoRI insert from pT267 (Pentecost and Teng, 1987); b) the 540bp mouse ß-casein Pst I insert from pBR322-mu-casein (Li et al, 1987); c) the 2.1 Kb human LTF EcoRI insert from pHLF1212 (Panella et al., 1991); d) the 210bp murine histone 3.2 SalI insert from pm His 3.2 (Marzluff and Graves, 1984).

RESULTS

Induction of lactoferrin gene expression in cultured mouse mammary epithelial cells.

LTF mRNA expression was not observed in CID-9 and SCp2 cells harvested from subconfluent cultures on plastic in either 5% serum containing or serum-free medium (Fig.1A, B). Tissue-specific expression of milk protein genes in these cell lines, and in primary and secondary mouse mammary epithelial cells, has previously been shown to depend on lactogenic hormones and contact with reconstituted basement membrane (Barcellos-Hoff et al., 1989; Desprez et al., 1993; Schmidhauser et al., 1992; Streuli et al., 1991). In order to determine the effect of basement membrane on LTF mRNA expression in mammary epithelium, CID-9 and SCp2 cells were

cultured in the presence or absence of complete reconstituted basement membrane (EHS-matrix) or purified laminin, a major basement membrane component. LTF mRNA expression could be induced in CID-9 cells by plating the cells on EHS matrix and in SCp2 cells by adding 50μg/ml of purified laminin to the culture medium. Unlike the caseins (Barcellos-Hoff et al., 1989, Schmidhauser et al., 1990), the expression of LTF was independent of the mammotrophic hormone, prolactin.

After SCp2 cells were grown to confluence on plastic in 5% serum containing medium, LTF mRNA was expressed without addition of exogenous basement membrane, suggesting that increased cell-cell interactions or cell shape changes due to crowding could replace the conditions for LTF induction provided by basement membrane. In this regard, the conditions for LTF expression differed from those for β-casein, where crowding per se is not sufficient and continued addition of basement membrane is required.

Cell rounding in the absence of cell-ECM or cell-cell interaction is sufficient for lactoferrin expression.

Having determined that high density cultures of SCp2 cells express LTF mRNA, we examined the roles of cell-cell interaction and cell shape changes in this inductive process by culturing SCp2 cells on polyHEMA coated substrata in the absence of serum or exogenous ECM. The neutrally charged plastic polymer, polyHEMA, prevents cell-substratum attachment and spreading. Under these conditions SCp2 cells aggregate to form rounded cell clusters in suspension and LTF mRNA expression is induced (Fig. 1C). LTF mRNA was expressed under these conditions as long as insulin was present in the serum-free medium. Hydrocortisone, while not absolutely necessary, elevated the level of LTF mRNA expression when added.

In order to distinguish between decreased cell-substratum adhesion and altered cell-cell interactions as the driving force behind the induction of LTF expression, we lowered the levels of extracellular Ca^{2+} so that calcium-dependent intercellular interactions would be decreased. Lowering the extracellular calcium concentration to 50μM in suspension cultures decreased the

number of large cell clusters and increased the proportion of single cells. In adherent cultures, lowering the extracellular calcium concentration to 50μM reduced the extent of cell spreading and gave the cells a more refractile appearance. In both suspended and attached cultures, lowered calcium levels were accompanied by increased LTF mRNA expression. Thus, changes in cell shape, rather than increased cell-cell cohesion appear to be sufficient for LTF expression.

In order to show conclusively that cell-cell contact is not required and that cell rounding alone is sufficient to trigger LTF expression, we asked whether LTF protein expression could be induced in single, suspended, mammary epithelial cells in the absence of either ECM or intercellular contact. LTF protein expression was compared immunocytochemically in suspended SCp2 cells cultured on polyHEMA in physiological (2mM) or in low (50μM) Ca^{2+} media, and in adherent SCp2 cells on glass coverslips in normal (2mM) Ca^{2+}media. LTF protein expression was apparent in single cells harvested from polyHEMA cultures but not in cells cultured on glass (Fig. 2).

Accumulation of lactoferrin mRNA occurs over 48 hours of suspension culture.

In order to determine the timecourse of LTF mRNA induction following release from a flattened, adherent state , SCp2 cells cultured on plastic were trypsinized and re-plated back onto either plastic or polyHEMA for varying times. Trypsin treatment disrupts the adherence of the cells to plastic within minutes. However, the SCp2 cells required 48 hours of culture on polyHEMA to achieve significant levels of LTF mRNA expression (Fig. 3). Cells replated onto plastic did not exhibit transient LTF mRNA expression at any of the time points examined. Hybridization of the same northern blot to a histone probe, which is used as a marker of proliferation, showed an inverse correlation with LTF expression.

Lactoferrin expression is induced in human mammary epithelial cells cultured on basement membrane or in suspension.

LTF mRNA is abundantly expressed in normal non-lactating human breast epithelium in

vivo and marked changes in expression are a common occurrence in pre-malignant and malignant breast tissue, suggesting that LTF could be used as a marker of breast function that is dysregulated in malignancy (Campbell et al., 1992). When cultured in contact with basement membrane, human breast epithelial cells from normal tissue have been previously reported to exhibit several aspects of the development and differentiation process, including formation of three-dimensional alveolar-like structures, basal deposition of endogenous basement membrane and apical secretion of sialomucins, (Stampfer and Yaswen, 1992; Petersen et al., 1992; Blaschke et al., 1995).

In order to investigate whether the signalling requirements for LTF expression in normal HMEC strains were similar to those observed in the murine cell lines, we compared organoids, isolated from surgically removed reduction mammoplasty tissue, and early passage cells, derived from the organoids, cultured on plastic, EHS-matrix and polyHEMA. LTF mRNA is abundantly expressed in organoids, but is downregulated in HMECs cultured on plastic (Fig. 4). In contrast, under identical media conditions, in the absence of added prolactin, 4^{th} passage HMECs plated on EHS-matrix show robust expression of LTF mRNA. In the absence of EHS-matrix, LTF mRNA is also expressed in 5^{th} passage HMECs that are forced to round up by culture on polyHEMA coated dishes. We have also observed LTF mRNA expression in cultures of HMEC allowed to grow to superconfluence (data not shown). Thus far, these results have only been obtained with early passage HMEC originally grown in serum containing medium. We have not observed LTF expression in the stem cell-like HMEC population which has long term growth potential in serum-free medium (Taylor-Papadimitriou and Stampfer, 1992). In sum, these data suggest that the cell shape dependent regulation of LTF expression in normal early passage human breast epithelial cell strains from non-pregnant, non-lactating tissue is similar to that of the mouse mammary epithelial cell lines derived from mid-pregnant mice.

DISCUSSION

Analysis of the role of microenvironment in directing cell function in culture has shown that ECM in general, and basement membrane in particular, is a potent regulator of tissue specific

growth, differentiation and morphogenesis in many cell types (for reviews see Adams and Watt, 1993; Stoker et al., 1990). In mammary epithelia, signals from basement membrane, in conjunction with hormones, are required for maintenance of complex three-dimensional alveoli and milk secretion. Biochemical and mechanochemical signals from ECM are integrated with those from soluble factors and other cells to coordinate differentiated function. Each differentiated function appears to depend on a distinct set of microenvironmental determinants (Lin and Bissell, 1993).

In this report, we have analyzed the signaling requirements of the iron binding protein, LTF, a secreted product of mammary epithelial cells, and a component of milk. We show that the expression of mouse and human LTF mRNA is modulated by ECM, but that this regulation is distinct from that of other milk proteins such as β-casein and WAP. Functional mouse CID-9 cells, their clonal derivatives - SCp2 cells, and early passage human mammary epithelial cells, cultured on plastic in either serum-containing or serum-free medium, did not express LTF mRNA, provided the cultures were maintained at sub-confluent density. When cultured on reconstituted basement membrane, these cells recovered the ability to express LTF mRNA. In CID-9 and SCp2 cells, LTF mRNA could be induced by overlay with pure laminin, the major glycoprotein constituent of EHS-matrix. In this regard, the modulation of LTF expression by substratum resembled that for β-casein. However, unlike the case for β-casein expression, we observed high levels of LTF mRNA expression at high cell densities in the absence of exogenous ECM administration. In addition, when the role of lactogenic hormones was assessed, we found that while β-casein expression was absolutely dependent on prolactin, LTF expression was prolactin insensitive. This finding is in apparent conflict with an earlier study on mouse mammary gland explants (Green and Pastewka, 1978), but is consistent with previous observations on bovine mammary epithelial cells cultured on collagen gels (Schanbacher et al., 1993; Talhouk et al., 1993). A possible explanation for this discrepancy between observations on tissue explants and cells in monolayer could be the presence of paracrine factors in the tissue explants which are not present in the isolated cell cultures.

Under all the conditions in which LTF expression was observed, we noticed an

accompanying change in cell shape, due either to passive cell crowding or increased cell cohesion. We explored this cell shape phenomenon further by using poly(HEMA) treated culture dishes to prevent cell attachment and spreading. Regardless of initial plating density, cells maintained in medium containing physiological calcium levels on poly(HEMA) treated plates formed tight aggregates and expressed abundant LTF mRNA. In order to distinguish between changes in cell shape and increased cell cohesion as driving forces for LTF induction, we lowered extracellular calcium levels so that cadherin dependent intercellular cohesion would be decreased. High levels of LTF mRNA were detected in cells maintained in low Ca^{2+} medium on plastic or poly(HEMA), suggesting that decreased cellular adhesion which results in cell rounding is sufficient to trigger LTF expression. We confirmed by immunocytochemistry that single rounded SCp2 cells, isolated from suspension cultures, could accumulate LTF protein, while cells that were well spread on plastic did not express LTF protein. Thus, although exogenous ECM is capable of supporting LTF expression, it is possible to circumvent the ECM effect and induce LTF directly by manipulating cell shape. These data suggest that ECM can modulate LTF expression solely by modifying cell shape, thereby altering a mechanochemical signalling pathway.

Modulation of cell shape is a well known mechanism by which cellular function can be regulated by ECM (reviewed in Ben-Ze'ev, 1991; Ingber, 1993; Singhvi et al., 1994; Watt, 1986). Different cell types may adopt different shapes depending on the ECM ligand encountered; Sertoli cells, for example, assume a columnar morphology while Schwann cells become elongated on laminin substrata (Kleinman et al., 1984). Alternatively one cell type may adopt different shapes in response to different ECM ligands; chondrocyte morphology is polygonal on plasma fibronectin but elongated on cellular fibronectin (West et al., 1984; West et al., 1979). Early evidence to suggest that cell shape changes may have a functional significance came from experiments designed to force cell shape changes independently of exposure to ECM. Under these circumstances, changes in morphology caused by depriving cells of substratum contact were shown to directly influence both cellular growth and differentiation (Folkman and Moscona, 1978; Gospodarowicz et al., 1978; Allan and Harrison, 1980; Glowacki et al., 1983; Shannon and

Pitelka, 1981; Watt et al., 1988; Rana et al., 1994). More recently, it was shown that ECM controls the growth of endothelial and hepatic cells by regulation of cell spreading (Ingber, 1990; Mooney et al., 1992) , and selectively stimulates the transcription of hepatocyte specific genes by promoting cell rounding (DiPersio et al., 1991).

In vivo, the changing ECM components of the mammary gland during different stages of growth and differentiation may provide different physical constraints on the shapes of responsive cells. These physical constraints may, in turn, dictate structural configurations of protein complexes on cell surfaces. Signals provided by ECM are known to be transduced by integrins, which are α/β heterodimeric transmembrane receptors (reviewed by Hynes, 1992). Ligation of integrins can initiate intracellular biochemical events, including flux in calcium and cAMP levels, activation of the Na^+/K^+ antiporter, and increases in tyrosine phosphorylation that propagate the ECM signal within the cell (Hynes, 1992; Juliano and Haskill, 1993; Schwartz et al., 1991). More recently, integrins have been found to operate as mechanochemical receptors which transmit mechanical signals across the cell membrane to the cytoskeleton (Schwartz and Ingber, 1994). Experiments are currently in progress in our laboratory to determine whether specific integrins are involved in cell shape dependence of LTF gene expression, and how changes in cell shape are translated into changes in LTF mRNA abundance within responsive cells.

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FIGURE LEGENDS

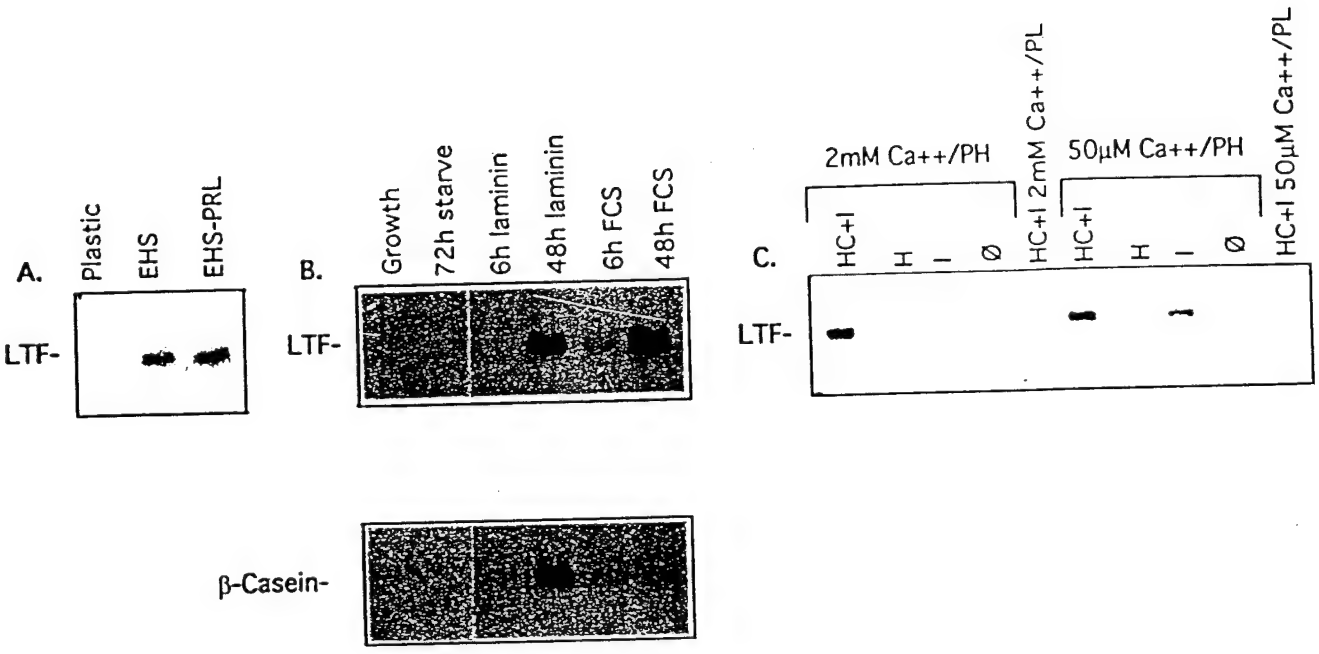
Figure 1 Northern analyses of total RNA extracted from (A) CID-9 or (B, C) SCp2 mouse mammary epithelial cells grown under different conditions. In (A) CID-9 cells were plated on plastic (Plastic) or reconstituted basement membrane (EHS) in the presence or absence (-PRL) of prolactin. In (B) SCp2 cells were plated on plastic at subconfluent cell density with 5% fetal calf serum (Growth), fed serum free medium for 72 hours (72h starve), fed serum free medium for 72 hours followed by medium supplementation with 50μg/ml laminin for 6 and 48 hours, or fed 5% FCS for 6 and 48 hours. In (C) SCp2 cells were plated on plastic (PL) or PolyHEMA (PH) coated plates in media containing physiological (2mM) or low (50μM) extracellular calcium concentrations, and no hormones (Ø), insulin (I), hydrocortisone (H), or hydrocortisone and insulin (HC+I). The cDNA probes used were mouse lactoferrin (LTF) and mouse ß-casein.

Figure 2 Staining of nuclei with 4,6-Diamidino-2-phenylindole (DAPI; Panels A,B,C) and immunolocalization of lactoferrin protein (Panels D,E,F) in adherent (Panels A and D) or suspension (Panels B and C, D and F) cultures of SCp2 cells in physiological (2mM; Panels A,B,D,E) or low (50μM; Panels C and F) calcium medium. Suspended cells were immoblized on polylysine coated coverslips prior to fixation. Lactoferrin expression was demonstrated by immunofluorescence using a polyclonal antibody against lactoferrin. Bar = 28.5 μ

Figure 3 Timecourse of lactoferrin mRNA induction in cells transferred from adherent to suspension culture. Growing cultures of adherent SCp2 cells were trypsinized and replated onto plastic (PL) or PolyHEMA (PH) coated surfaces at 0 h. Total RNA was harvested from matched cultures at 0, 12, 24 and 48 h and subjected to northern analysis using lactoferrin (LTF) and histone cDNA probes.

Figure 4 Northern analysis of lactoferrin mRNA expression in epithelial organoids purified from surgically removed reduction mammoplasty tissue and cultured normal human mammary epithelial cells. Total RNA was extracted from primary organoids as well as 4th and 5th passage cultures of human mammary epithelial cells grown on plastic, reconstituted basement membrane (EHS) or PolyHEMA coated plastic. The RNA samples were subjected to northern analysis using a lactoferrin (LTF) cDNA probe. The total RNA in the original gel was stained with Ethidium Bromide (EtBr) for comparison of RNA loading.

Figure 1


A.
Plastic
EHS
EHS-PRL
LTF-
B.
Growth
72h starve
6h laminin
48h laminin
6h FCS
48h FCS
LTF-
β-Casein-
C.
2mM Ca++/PH
HC+I
H
–
Ø
HC+I 2mM Ca++/PL
50μM Ca++/PH
HC+I
H
–
Ø
HC+I 50μM Ca++/PL
LTF-

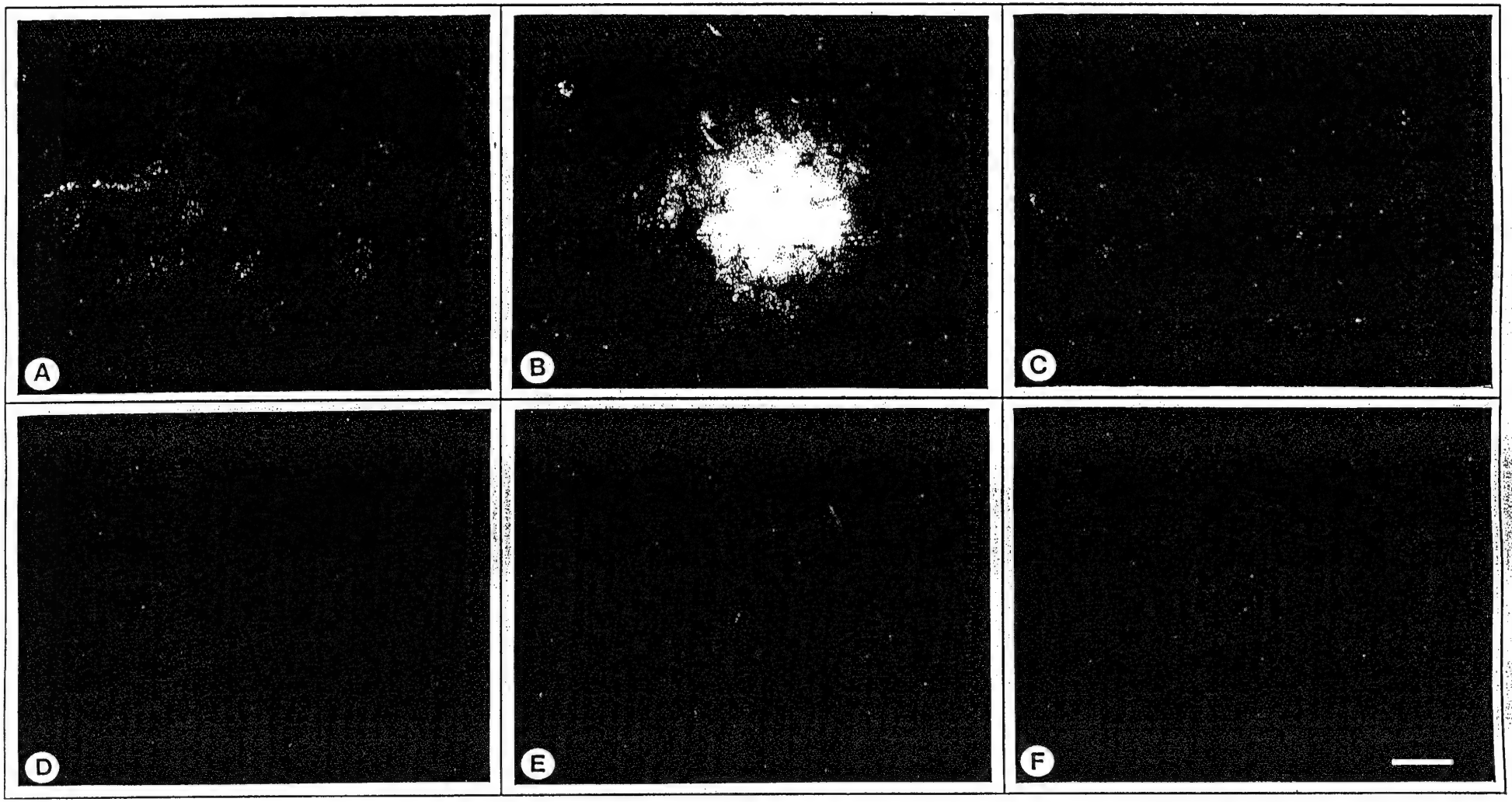


Figure 2

Figure 3

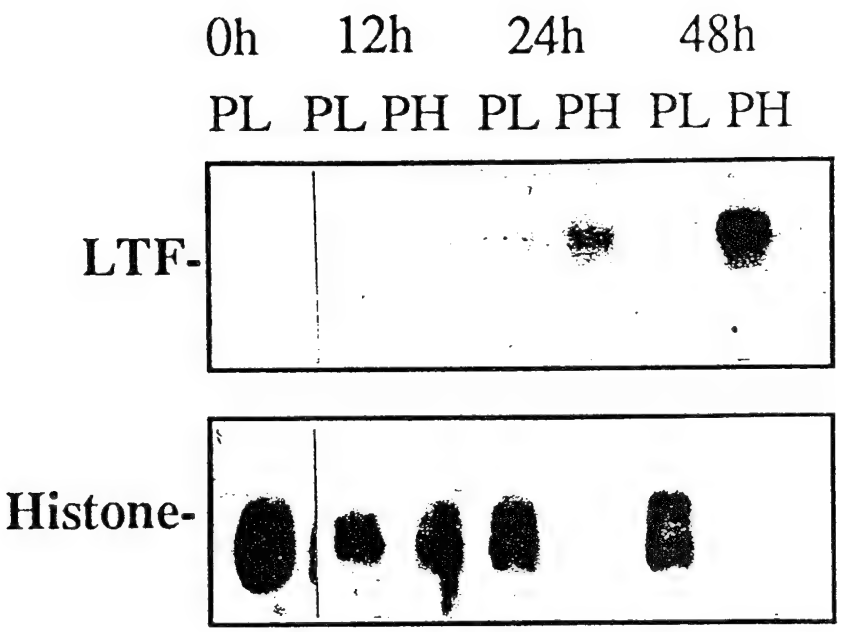
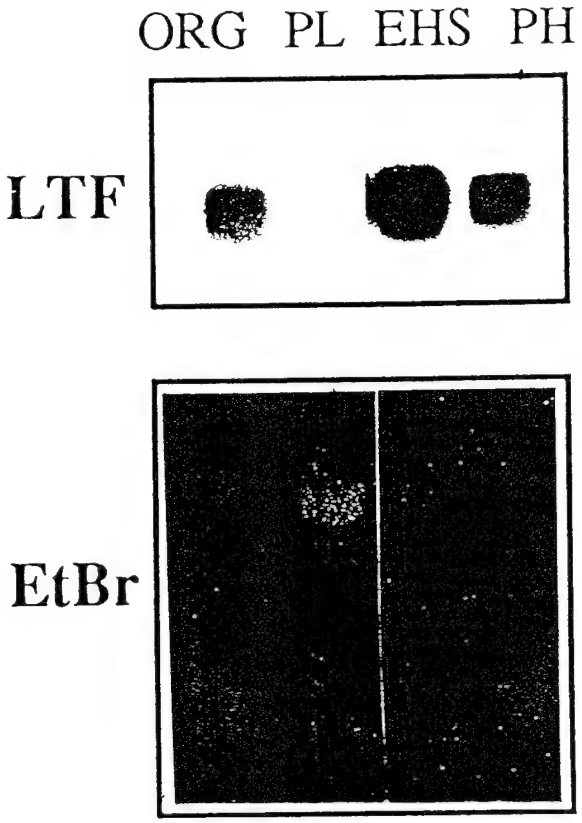


Figure 4



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Cellular growth and survival are mediated by β1 integrins in normal human breast epithelium but not in breast carcinoma

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SUMMARY

We previously established a rapid three-dimensional assay for discrimination of normal and malignant human breast epithelial cells using a laminin-rich reconstituted basement membrane. In this assay, normal epithelial cells differentiate into well-organized acinar structures whereas tumor cells fail to recapitulate this process and produce large, disordered colonies. The data suggest that breast acinar morphogenesis and differentiation is regulated by cell-extracellular matrix (ECM) interactions and that these interactions are altered in malignancy. Here, we investigated the role of ECM receptors (integrins) in these processes and report on the expression and function of potential laminin receptors in normal and tumorigenic breast epithelial cells. Immmunocytochemical analysis showed that normal and carcinoma cells in a three-dimensional substratum express profiles of integrins similar to normal and malignant breast tissues in situ. Normal cells express α1, α2, α3, α6, β1 and β4 integrin subunits, whereas breast carcinoma cells show variable losses, disordered expression, or downregulation of these subunits. Function-blocking experiments using inhibitory anti-integrin subunit antibodies showed a >5-fold inhibition of the formation of acinar structures by normal cells in the presence of either anti-β1 or anti-α3 antibodies, whereas anti-α2 or -α6 had little or no effect. In experiments where collagen type I gels were used instead of basement membrane, acinar morphogenesis was blocked by anti-β1 and -α2 antibodies but not by anti-α3. These data suggest a specificity of integrin utilization dependent on the ECM ligands encountered by the cell. The interruption of normal acinar morphogenesis by anti-integrin antibodies was associated with an inhibition of cell growth and induction of apoptosis. Function-blocking antibodies had no inhibitory effect on the rate of tumor cell growth, survival or capacity to form colonies. Thus under our culture conditions breast acinar formation is at least a two-step process involving β1-integrin-dependent cellular growth followed by polarization of the cells into organized structures. The regulation of this pathway appears to be impaired or lost in the tumor cells, suggesting that tumor colony formation occurs by independent mechanisms and that loss of proper integrin-mediated cell-ECM interaction may be critical to breast tumor formation.

Key words: mammary epithelium, extracellular matrix, basement membrane, integrin, growth, apoptosis

INTRODUCTION

It is now widely accepted that extracellular matrix is a key component of tissue microenvironment and a determinant of functional differentiation in developing and adult epithelia (Stoker et al., 1990; Adams and Watt, 1993; Hay, 1993). In the mammary gland, extensive data exist showing that basement membrane components regulate the morphological and functional differentiation of mammary epithelial cells in culture and in vivo (Streuli et al., 1991; Petersen et al., 1992; Shearer et al., 1992; Sympson et al., 1994; for review see Howlett and Bissell, 1993). In addition, altered interactions with ECM have been observed in mammary tumor development, emphasizing the importance of microenvironmental regulation in normal development and malignancy (Chiquet-Ehrismann et al., 1986; for reviews see Haslam, 1991; Roskelley et al., 1993).

We recently established an assay for analyzing the mechanisms by which normal and malignant human mammary epithelial cells are regulated by microenvironmental cues in culture (Petersen et al., 1992). In this assay, when normal cells from twelve reduction mammoplasties and two normal cell lines were embedded in a reconstituted basement membrane as single cells, they underwent a process of morphogenesis to generate small, well-differentiated acinar structures that frequently deposited an endogenous basement membrane, secreted sialomucins apically, and stopped growing. In contrast, tumor cells from six established breast carcinoma cell lines and two breast carcinoma specimens failed to recapitu-

late this process and produced large, disorganized, poorly differentiated colonies and as far as the cell lines were concerned grew continuously. These data indicate the importance of cell-basement membrane interactions in the regulation of normal breast differentiation and point to a loss of this regulation in tumorigenesis. Since the two contrasting phenotypes expressed by normal and tumor cells are apparent only in the context of a physiologically relevant microenvironment (i.e. three-dimensional basement membrane) we hypothesized that correct cell-ECM interactions are suppressive for the expression of aberrant growth and differentiation characteristics. In other words, we proposed that as breast tumor cells manifest their malignant phenotype, they lose the capacity to respond correctly to information provided by the ECM.

Signals provided to mammary epithelium by basement membrane may be mediated by integrins, the transmembrane heterodimeric cell-surface receptors that link ECM to structural and functional elements within the cell (Hynes, 1992; Damsky and Werb, 1992; Juliano and Haskill, 1993). Evidence from the mouse mammary gland indicates that the expression of the mammary-specific gene β-casein is triggered by signals from basement membrane, specifically conferred by elements of the laminin molecule (Streuli et al., 1991, 1995). The ECM signal is transduced through the β1 integrin family, as evidenced by the ability of function-blocking anti-β1 integrin antibodies to block the expression of β-casein (Streuli et al., 1991). With the advent of reliable criteria for discriminating normal and malignant human breast epithelial cells using basement membrane (Petersen et al., 1992), it is now possible to analyze the mechanisms of tumor cell responsiveness to basement membrane in relation to normal cells and the role of integrins in this process. Several integrin receptors for laminin are expressed in normal human mammary epithelium. These include α1β1, α2β1, α3β1, α6β1 and α6β4. Altered expression of these receptors is a common occurrence in breast tumors (Zutter et al., 1990; Koukoulis et al., 1991; D'Ardenne et al., 1991; Pignatelli et al., 1991, 1992; Jones et al., 1992; Natali et al., 1992; Zutter et al., 1993; Berdichevsky et al., 1994), implying that loss or downregulation of integrins may be critical to the development of malignancy by altering ECM-induced differentiation. It is not possible to conclude from surveys such as those mentioned above which of the many integrins expressed may be functionally active nor which change(s) in integrin expression may be of consequence in tumorigenesis.

We have used our reconstituted basement membrane assay to investigate the mechanisms of normal breast and breast-carcinoma cell interactions with basement membrane and have analyzed the expression and function of several integrins that recognize laminin. We report that: (1) normal cells and tumor cells express profiles of laminin-binding integrin receptor subunits that are similar, respectively, to those of normal and malignant breast tissue in situ; (2) cell survival, growth, and morphogenesis of acinar structures by normal cells is integrin dependent; and (3) the formation of disordered colonies by tumor cells occurs by pathways that are independent of those utilized for formation of acinar structures.

MATERIALS AND METHODS

Substrata and antibodies

EHS matrix was prepared from EHS ascites tumors passaged in C57BL mice at a concentration of 7-10 mg/ml and stored at 0-4°C for up to 4 weeks as described (Streuli et al., 1991; Blaschke et al., 1994). In some experiments, commercially prepared EHS matrix (Matrigel, Collaborative Research, Bedford MA) was used. Type I collagen was extracted from rat tail tendons as described (Lee et al., 1984) and stored at ~3 mg/ml in 0.1% acetic acid. The collagen solution was dialyzed 1 part collagen to 10 parts water with 3 changes over 48 hours at 4°C and equilibrated to 1× DME/F12 medium prior to use as described (Streuli et al., 1991).

Rabbit polyclonal antibodies to α1 (AB1934) and mouse monoclonal antibodies (mAbs) to α2 (clone P1E6 and clone CLB-150), α3 (clone P1B5), α6 (GoH3) and β1 (clone JB1a) were obtained from Chemicon International, Temecula CA. Mouse mAbs against β4 (clone 3E1) were obtained from Life Technologies, Gaithersberg MD. Rat mAbs to α5 (BII62; Werb et al., 1989), α6 (J1B5; Damsky et al., 1992) and β1 (AIIB2; Damsky et al., 1992) were also used. Mouse monoclonal anti-human type IV collagen (PHM-12) (AMD, Armaton, Australia) was used to assess endogenous basement membrane immunoreactivity as described previously (Petersen et al., 1992).

Cell culture

Early passage HMT-3522 (Briand et al., 1987) and MCF-10A (Soule et al., 1990) breast epithelial cells, which behave like normal primary cells from reduction mammoplasties when cultured within EHS (Petersen et al., 1992), served as a model for normal breast epithelium. These cells form small polarized acinus-like structures within EHS that growth arrest by 6-12 days of culture. Tumorigenic HMT-3909/S13, MCF-7 subline 9, and MDA-MB-435 cells were used as a model for breast carcinoma. These cells form large disordered colonies that grow continuously when cultured within EHS (Petersen et al., 1992; Howlett et al., 1994). The normal and carcinoma cells were initially cultured in monolayer as previously described (Petersen et al., 1992; Howlett et al., 1994). The carcinoma cells require type 1 collagen substrata for monolayer culture in serum-free medium (reviewed by Blaschke et al., 1995). The cells were then trypsinized and embedded in either 300 μl of EHS matrix or a gel of collagen type I, as single cells, at a concentration of approximately 2.5×10^5 cells per well of a 24-well plate and cultured as previously described (Petersen et al., 1992).

Immunohistochemistry

EHS cultures were fixed in either 2% paraformaldehyde at ambient temperature for 20 minutes or in 1:1 methanol:acetone at –20°C for 2-3 minutes. Specimens were embedded in sucrose, frozen in Tissue-Tek OCT compound (Miles Laboratories), and 5 μm frozen sections were prepared for immunostaining as described (Streuli et al., 1991). Sections were incubated with primary antibodies for 60 minutes followed by biotinylated secondary antibodies (30 minutes) and Texas Red-conjugated streptavidin (30 minutes). Control sections were stained with second antibodies only.

Integrin function-blocking assays

Antibodies against the α2 (clone P1E6), α3 (clone P1B5), α5 (clone BII62), α6 (GoH3), and β1 (clone AIIB2) integrin subunits, and control non-immune mouse and rat IgG were introduced into the cell-embedded substratum at the time of EHS and type I collagen gelation and into the medium at all subsequent medium changes at a range of concentrations from 10-100 μg/ml.

At the end of experiments, cultures were scored for the capacity of normal cells to produce mature acinar structures and the tumor cells to produce colonies. Ten random fields were viewed microscopically and the numbers of normal spheres or tumor colonies were counted in each field. The numbers of spheres/colonies formed in the presence of antibodies were calculated as a percentage of those formed by control cultures without antibodies.

Analysis of cellular growth and apoptosis

The growth capacity of cells was determined by 24-hour incorpora-

tion of [^{3}H]TdR (20 Ci/mmol, NEN Research Products, Dupont) and determination of thymidine-labeling indices in 5 μm frozen sections as described (Petersen et al., 1992).

Apoptosis was assessed in frozen sections by detection of FITC-digoxigenin nucleotide labelling of 3′OH DNA ends using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersberg, MD). Thymocytes isolated from human peripheral blood were used as controls. Nuclei were counterstained with DAPI as described (Streuli et al., 1991).

Experimental subjects

In conducting research using animals, the investigators adhered to the 'Guide for the Care and Use of Laboratory Animals', prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH publication no. 86-23, revised 1985). In the conduct of research where humans were the subjects, the investigators adhered to the policies regarding the protection of human subjects as prescribed by 32 CFR 219 and subparts B, C and D.

RESULTS

The profile of integrin subunits expressed by normal and tumorigenic human breast epithelial cells in vivo is recapitulated in three-dimensional culture

To determine whether normal and tumor cells, cultured within reconstituted basement membrane, express a profile of integrins similar to that shown in vivo, normal HMT-3522 cells and tumorigenic HMT-3909/S13, MCF-7 subline 9 (MCF-7/9), and MDA-MB-435 breast carcinoma cells were cultured in EHS matrix for 12 days. Cultures were examined for the expression of integrin subunits by immunocytochemistry. The normal and tumorigenic cell lines were found to broadly recapitulate the pattern of integrin subunit expression and localization predicted from published in vivo data (Fig. 1 and Table 1). In normal HMT-3522 cells, weak staining for α1 was seen in the cytoplasm, and for α2 at the basolateral surface. Staining for the α3 and β1 subunits was strong, and was localized primarily to the basolateral surface with some expression within the cytoplasm (Fig. 1A,D). Staining for the α6 and β4 subunits was also strong, but the signal was restricted primarily to the basal cell surface (Fig. 1G,J). In contrast, HMT-3909/S13, MCF-7/9 and MDA-MB-435 breast carcinoma cells showed loss, disordered expression, or downregulation of these integrin subunits, and the changes appeared to correlate with the level of tumor aggression in that disordered integrin expression was most pronounced in the metastatic MDA-MB-435 cells (Fig. 1; Table 1). All tumor cells expressed α1 to varying degrees. Staining for α2, α6, and β4 subunits was not detected on the metastatic MDA-MB-435 cells, and staining for α3 and β1 was reduced. The breast carcinoma lines HMT 3909/S13 and MCF-7/9 cells showed disordered expression and/or losses of these subunits. Staining for α2 and α6 was confirmed with P1E6 and CBL-150 (α2) and J1B5 and GoH3 (α6) antibodies in both normal and carcinoma cells.

Formation of acinar structures within EHS is integrin dependent

To determine which of the various integrins expressed by human breast epithelial cells are functionally relevant to the formation of acinar structures, specific inhibitory anti-integrin antibodies were used to interfere with this process in reconstituted basement membrane culture. Normal HMT-3522 cells cultured in EHS without antibodies, or with 10 μg/ml or 100 μg/ml of non-immune mouse or rat IgG, formed well-organized acinar structures at similar frequencies, although slight increases were noted for non-immune IgGs (Table 2). In contrast, inhibitory anti-β1 subunit antibodies, at similar concentrations, severely impaired the formation of spheres by HMT-3522 cells relative to control cultures; occasional spheres were observed scattered throughout the gels, but the vast majority of the cells introduced into the EHS gels remained as suspended single cells (Fig. 2 and Table 2). These effects were observed with two different anti-β1 antibodies and were dose dependent: mouse anti-β1 mAb (JB1a) induced a two-fold inhibition of sphere formation at 40 μg/ml and a 4-fold inhibition at 200 μg/ml, whereas rat anti-β1 mAb (AIIB2) caused an almost complete inhibition at 100 μg/ml (Table 2; Fig. 2). Similar results were obtained at day 6 and day 12 of culture. These data suggest that sphere formation by normal human mammary epithelial cells in response to EHS is dependent on integrin(s) of the β1 integrin family.

To define more precisely which integrins were critical in signaling acinar morphogenesis, similar experiments were performed with inhibitory anti-α2, -α3, and -α6 antibodies. Anti-α3 reduced sphere formation by 50% at 10 μg/ml and by

Table 1. Summary of immunolocalization of integrin subunits in human breast epithelial cells cultured in EHS matrix

	Integrin subunit					
Cell line	α1 (AB1934)	α2 (P1E6; CLB-150)	α3 (P1B5)	α6 (J1B5; GoH3)	β1 (AIIB2)	β4 (3E1)
HMT-3522	±*	±†	+++*,†	++++‡	+++†	++++‡
HMT-3909	+*	–	+++*,†	±*,‡	++++	–§
MCF-7/9	+*	–	±*,†	–¶	±	–
MDA-MB-435	±*	–§	±*	–§	±*	–§

*Cytoplasmic localization.
†Basolateral localization.
‡Basal localization.
§Some very weak cytoplasmic staining.
¶Weak cytoplasmic staining with J1B5 but mostly negative with GoH3.

Table 2. Effect of function-blocking anti-integrin antibodies on sphere formation by normal mammary epithelial cells

	Percentage of acinar structures formed within three-dimensional substrata					
Substratum	Non-immune mouse IgG	Non-immune rat IgG	Mouse anti-α2	Mouse anti-α3	Rat anti-α6	Rat anti-β1
EHS matrix	93.7±19.4	124±24	106.8±31	20.8±15	71.3±10	6.4±9
Collagen I	130±21.4	123±25.7	51.8±21	109±43	ND	6.3±8.3

Normal mammary cells (HMT-3522) were cultured in EHS or type 1 collagen matrix for a minimum of 6 days. The number of mature acinus-like spheres was scored microscopically in 10 random low power fields. Sphere frequency is expressed as a percentage relative to control cultures (no antibodies); values are means ± s.d. Anti-α2 (P1E6); anti-α3 (P1B5); anti-α6 (GoH3) and anti-β1 (AIIB2) integrin subunit antibodies and control Igs were used at 100 μg/ml. ND, not determined.

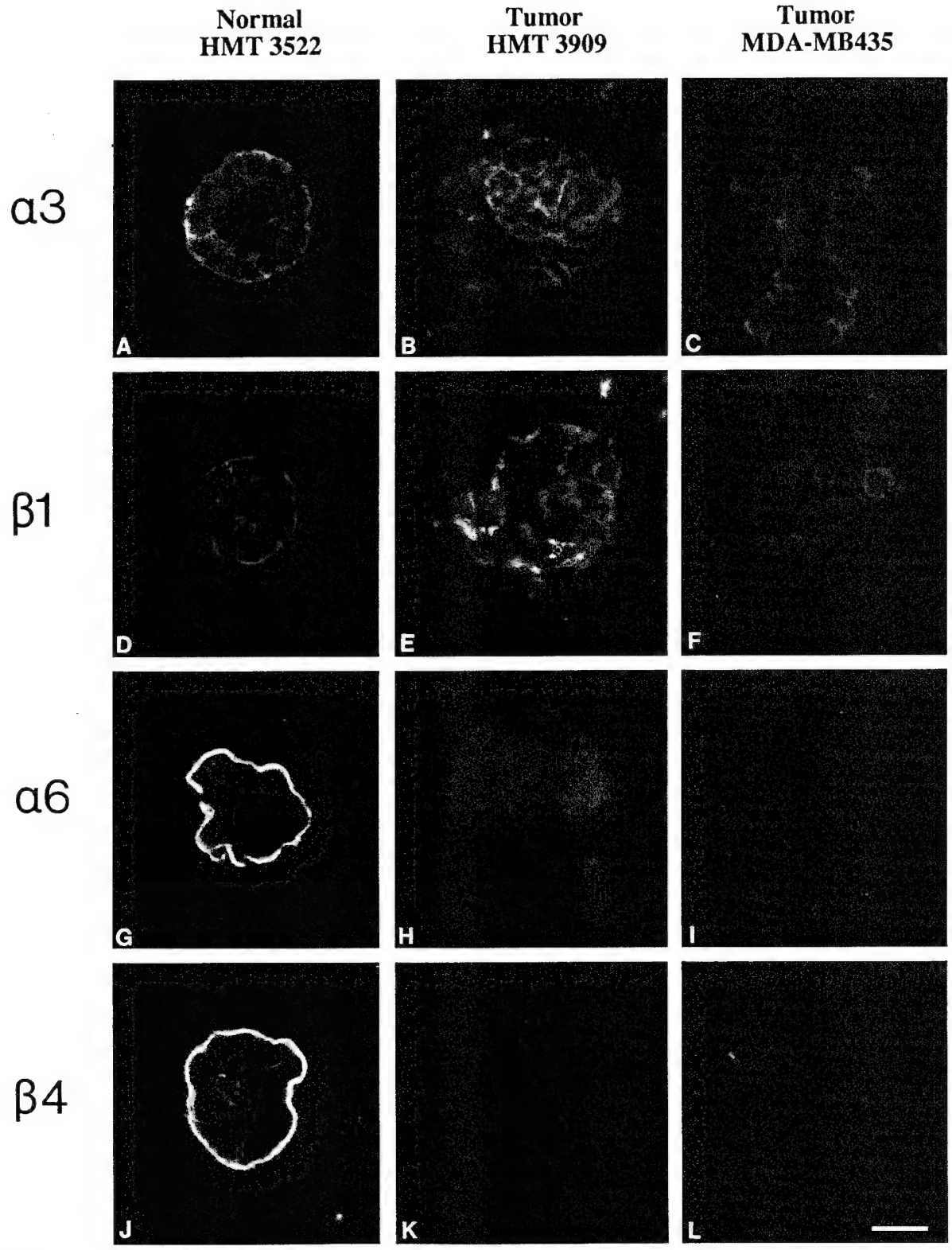

Fig. 1. Expression of laminin receptor integrin subunits by normal human mammary cells and tumor cells within EHS matrix. Normal and malignant human mammary epithelial cells were cultured within EHS for 12 days. The α3 (A-C), β1 (D-F), α6 (G-I), and β4 (J-L) integrin subunits were localized by immunofluorescence in 5 μm frozen sections using antibodies P1B5, AIIB2, J1B5 and 3E1, respectively. Normal HMT-3522 cells show basolateral expression of α3 and β1 (A,D), whereas the carcinoma cells show diffuse cytoplasmic localization of approximately equal (HMT-3909/S13, B,E) or greatly diminished (MDA-MB-435, C,F) intensity. Normal HMT-3522 cells show strong basal staining for α6 and β4 (G,J), whereas tumor cells show variable weak cytoplasmic staining (HMT-3909/S13, H,K) or almost no staining at all (MDA-MB-435, I,L). Bar, 21 μm.

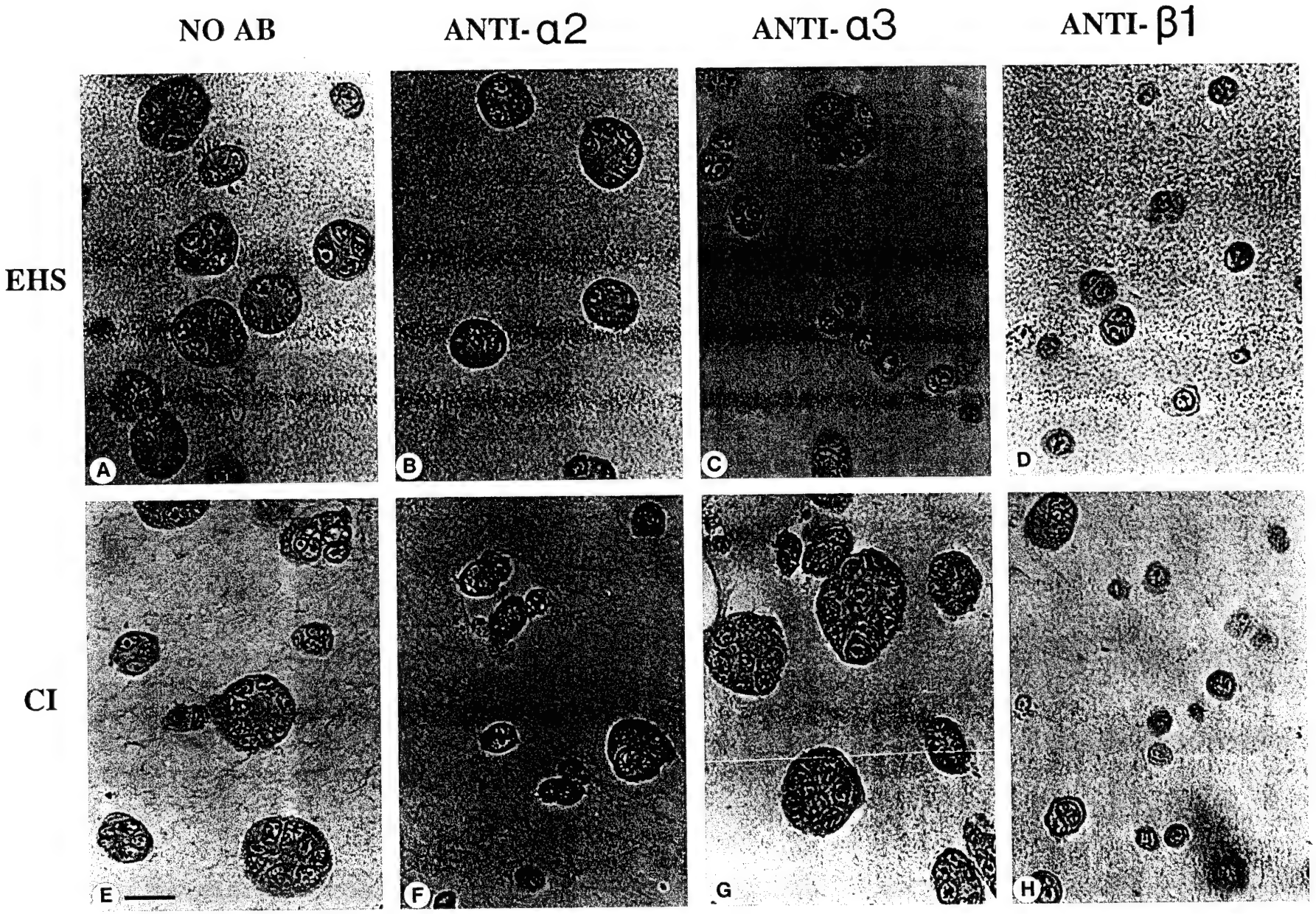

Fig. 2. Formation of acinar structures by normal HMT-3522 cells in both EHS and type 1 collagen gels by day 6 of culture (A,E). In the presence of 100 μg/ml inhibitory anti-β1 subunit antibodies (clone AIIB2) formation of acinus-like spheres is severly hampered (D,H). In EHS culture 100 μg/ml anti-α3 (clone P1B5), but not 100 μg/ml anti-α2 (clone P1E6), antibodies interfere with the formation of acini (B,D), whereas in collagen gels the reverse applies in that inhibitory anti-α2 blocks sphere formation but anti-α3 does not (F,G). Bar, 26 μm.

80% at 100 μg/ml. Anti-α5 and anti-α6 antibodies were much less effective (about 70% of control) at both 10 μg/ml and 100 μg/ml, while inhibitory anti-α2 antibodies showed no appreciable inhibition. These data indicate that, of the integrin subunits assayed, α3β1 appears to be the most significant in mediating morphogenesis in a basement membrane matrix. The extent of inhibition with anti-α3 antibodies was not equivalent to that with anti-β1, suggesting either that 100 μg/ml of anti-α3 antibodies was not sufficient to block or that additional β1 integrins may also be involved.

Normal HMT-3522 cells utilize alternative integrins for sphere morphogenesis in collagen type I gels

Normal mammary epithelial cells encounter basement membrane (BM) in vivo, and the data presented herein suggest that formation of acinar structures in response to BM requires α3β1 integrin. Previous studies have shown that the normal mammary cell line MTSV1-7 utilizes α2β1 integrin to form tubules and balls in response to collagen type I (Berdichevsky et al., 1992). We therefore analyzed the capacity of normal mammary cells to form spheres and deposit endogenous BM in type I collagen gels. Normal HMT-3522 cells produced well-ordered spheres in collagen gels as evidenced by morphology (Table 2; Fig. 2). However, although HMT-3522 cells basally deposited endogenous type IV collagen and laminin in reconstituted BM (as previously reported, Petersen et al., 1992), the cells did not deposit endogenous BM when cultured in type I collagen (Fig. 3). This suggests that although HMT-3522 cells are capable of forming spheres in both substrata, the level of organization of these structures is not equivalent.

The process of sphere formation by HMT-3522 cells in type I collagen gels was inhibited by 100 μg/ml of inhibitory anti-β1 antibodies to approximately the same extent as that found in EHS. Further analysis of the effects of inhibitory anti-α2 and α3, antibodies showed that in contrast to the data obtained for EHS cultures, 100 μg/ml of anti-α3 antibodies had no appreciable effect on sphere formation in collagen gels, but with anti-α2 approximately 50% inhibition was observed (Table 2; Fig. 2). Although this effect was analogous to α3 blocking in EHS, the α2 block in collagen was weaker than the β1 block and also was variable. These data suggest that β1 integrins mediate sphere formation by HMT-3522 cells in collagen gels as they do in EHS but that a different member of the β1 family (α2β1 instead of α3β1) may be preferentially used to respond to collagen.

The inhibition of acinar morphogenesis by anti-integrin antibodies is associated with an inhibition of cell growth

Previous studies with normal mammary epithelial cells using analysis of thymidine-labeling indices (TLI) have shown that an initial burst of cell growth is followed by organization of the cells into well-differentiated acinar structures whose growth is arrested by day 7-12 of culture (Petersen et al., 1992). The data presented in this report show that acinar formation is integrin dependent. Since normal mammary cells seeded into EHS in the presence of inhibitory anti-integrin antibodies remain suspended as single cells for the duration of the experiments, we asked whether the inhibitory antibodies interfered with acinar formation by blocking cellular growth. The

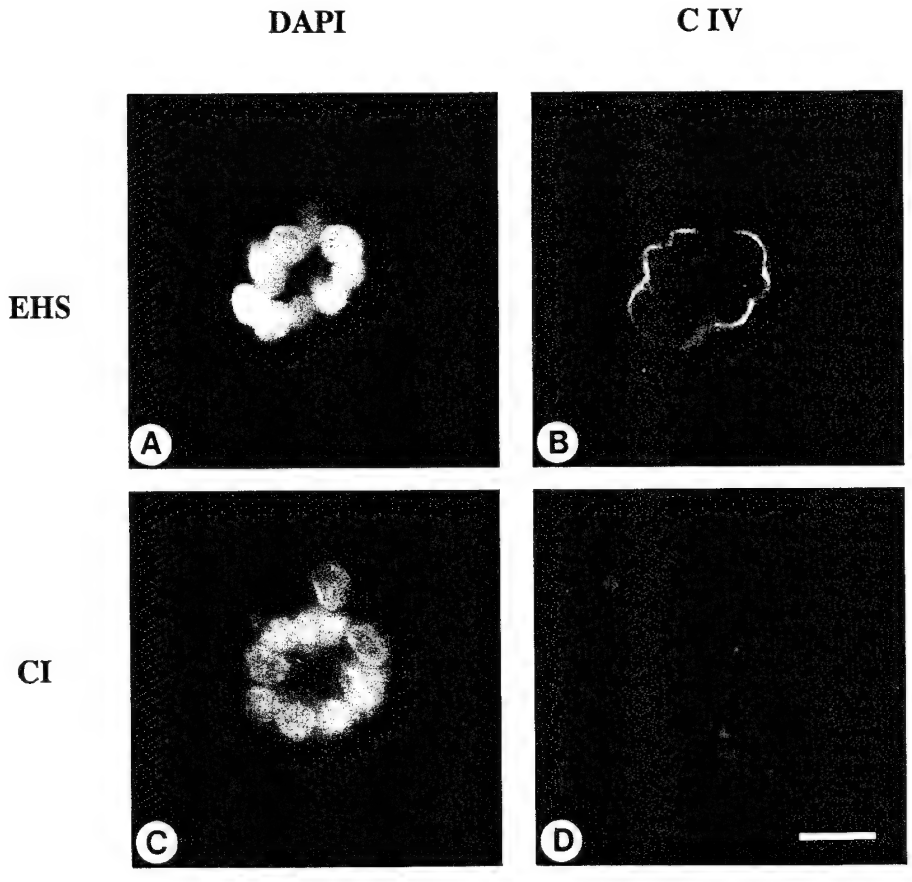

Fig. 3. Expression of endogenous basement membrane by normal HMT-3522 cells cultured within reconstituted basement membrane (EHS) and type 1 collagen (CI) gels. HMT-3522 cells were cultured within 3-dimensional substrata for 12 days. 5 μm frozen sections were double stained with DAPI to visualize nuclei (A,C) and with antibodies specific for human type IV collagen (CIV; B,D) to assess endogenous basement membrane immunoreactivity. Note that HMT-3522 cells form acinar structures in both substrata but basally deposit endogenous basement membrane in reconstituted BM only (B). Bar, 18 μm.

thymidine-labeling indices of normal HMT-3522 cells in both EHS and collagen were determined at day 2 and day 6 of culture.

In the absence of inhibitory anti-β1 antibodies the TLIs were approximately 60% at day 2 in both substrata but fell to 8.8% in collagen and 3% in EHS by day 6 as the cells formed differentiated acini. In contrast, in the presence of inhibitory anti-β1 antibodies the cells remained suspended as single cells, and the TLIs were low at day 2 in both substrata and remained low throughout the experiment (Table 3; Fig. 4). At day 6 of culture in both substrata, the low TLIs observed in the presence of anti-β1 antibodies reflect a small number of growth-arrested differentiated cells that formed acini by escaping the antibody blockade, and a majority of growth-inhibited single cells blocked by anti-integrin antibodies. These data suggest that an initial phase of cell growth is a requirement for acinar formation in three-dimensional culture. Thus, acinar formation appears to be a two-step process involving a β1-integrin-dependent cellular growth phase, followed by a phase of cell polarization to form the final organized structures.

Interruption of normal mammary cell-basement membrane interactions induces apoptosis

Prevention of appropriate cell-ECM contact by use of non-adhesive (polyHEMA) coated substrata, ECM fragments or RGD peptides can inhibit cell growth and differentiation in anchorage-dependent cells (Hayman et al., 1985; Ingber, 1990) and trigger programmed cell death or apoptosis (Frisch and Francis, 1994). Data presented herein show that inhibition of mammary cell attachment to basement membrane by ligation of β1 integrins blocks cellular growth and acinar formation. We therefore asked whether the inhibition of mammary cell-BM interaction induces apoptosis. Normal HMT-3522 cells were embedded within EHS matrix in the presence or absence of function-blocking anti-β1 antibodies and assayed for evidence of apoptosis at day 2 and day 6 of culture by detection of FITC-digoxigenin nucleotide labelling of 3′OH DNA ends using the ApopTag in situ apoptosis detection kit.

In the absence of anti-β1 antibodies, HMT-3522 cells formed acinar structures as described above. Apoptotic nuclei were detected infrequently (0.74%) at day 2, whereas at day 6, 6.3% of single cells not incorporated into acini and 2.5% of individual cells within acini were stained with the ApopTag reagents (Fig. 5). In contrast, in the presence of anti-β1 antibodies, 20.9% of the cells contained nuclei stained by ApopTag reagents at day 2 and at day 6, 59.9% of the nuclei were labelled. Interestingly, a small number of acini (~6% of control) formed in the presence of inhibitory anti-β1 integrin (see Table 2). The cells within these acini did not contain nuclei stained with the ApopTag reagents.

These data suggest strongly that β1 integrins transmit signals from ECM that are required for survival.

Inhibitory anti-integrin subunit antibodies do not block colony formation by breast carcinoma cells in reconstituted basement membrane

To determine whether the formation of colonies by breast carcinoma cells in reconstituted basement membrane reflects a failure of the cells to sense BM correctly, the effects of inhibitory anti-integrin antibodies were tested on HMT-3909/S13 and MDA-MB-435 cells. We found that inhibitory anti-β1 antibodies did not inhibit the capacity of tumor cells to make colonies within EHS at 100 μg/ml; the concentration that inhibited acinar formation by normal cells (Table 4A). Furthermore, anti-α2, -α3, and -α6 antibodies were also ineffective, although in the presence of anti-α3 the MDA-MB-435 cells appeared to send out more cellular processes at the margins of the tumor colonies (data not shown).

We then asked whether inhibitory anti-integrin antibodies could influence the growth properties of breast carcinoma cells or induce apoptosis in three-dimensional culture. HMT-3909/S13, MDA-MB-435 and MCF-7/9 cells were cultured in Matrigel for 6 days in the presence or absence of anti-β1 integrin antibodies. Growth was assessed by [^{3}H]TdR autoradiography and apoptosis was evaluated by detection of FITC-digoxigenin nucleotide labelling of 3′OH DNA ends using the ApopTag in situ apoptosis detection kit. Control cultures (no antibodies) of HMT-3909/S13 and MDA-MB-435 carcinoma cells showed moderate TLIs of approximately 10%, whereas MCF-7/9 cells grew more aggressively, with a TLI of 25%. No inhibition of cellular growth was observed in the presence of inhibitory anti-β1 antibodies (Table 4B). In fact slight increases in labeling indices were noted for HMT-3909/S13

Table 3. Effect of function-blocking anti-β1 integrin antibodies on normal breast epithelial cell growth within three-dimensional substrata

	Thymidine-labeling index	
Substratum	Medium (no antibodies)	Anti-β1 integrin
Day 2		
EHS matrix	60±22	2.2±5.7
Collagen I	62±23	14.2±16
Day 6		
EHS matrix	3±3.5	2.5±7
Collagen I	8.8±2.5	0

Normal breast cells (HMT-3522) were cultured in EHS matrix and collagen I substrata for 2-6 days. Cultures were labeled with [^{3}H]thymidine on days 2 and 6 of culture. 5 μm frozen sections were processed for autoradiography. Values are means ± s.d. The inhibitory anti-β1 (AIIB2) integrin subunit antibody was used at 100 μg/ml.

Table 4. Effect of function-blocking ant-β1 integrin antibodies on colony formation and cellular growth of mammary carcinoma cells within reconstituted basement membrane

	Percentage of colonies*		Thymidine-labeling index†	
Cells	Non-immune IgG	Anti-β1 integrin	Medium (no antibodies)	Anti-β1 integrin
HMT-3909/S13	61	100	10.1±12.8	19.2±10.1
MDA-MB-435	88	127.4	9.0±5.0	7.0±1.4
MCF-7/9	ND	ND	25.0±8.6	43.4±5.9

*To assess colony formation, carcinoma cells were cultured in EHS matrix for a minimum of 6 days. The number of tumor colonies was scored microscopically in 10 random low-power fields. Colony frequency is expressed as a percentage relative to control cultures (no antibodies).

†To assess growth, carcinoma cells were cultured within Matrigel for 6 days. Cultures were labeled with [^{3}H]thymidine and 5 μm frozen sections were processed for autoradiography. Values are means ± s.d.

The inhibitory anti-β1 (AIIB2) integrin subunit antibody was used at 100 μg/ml. ND, not determined.

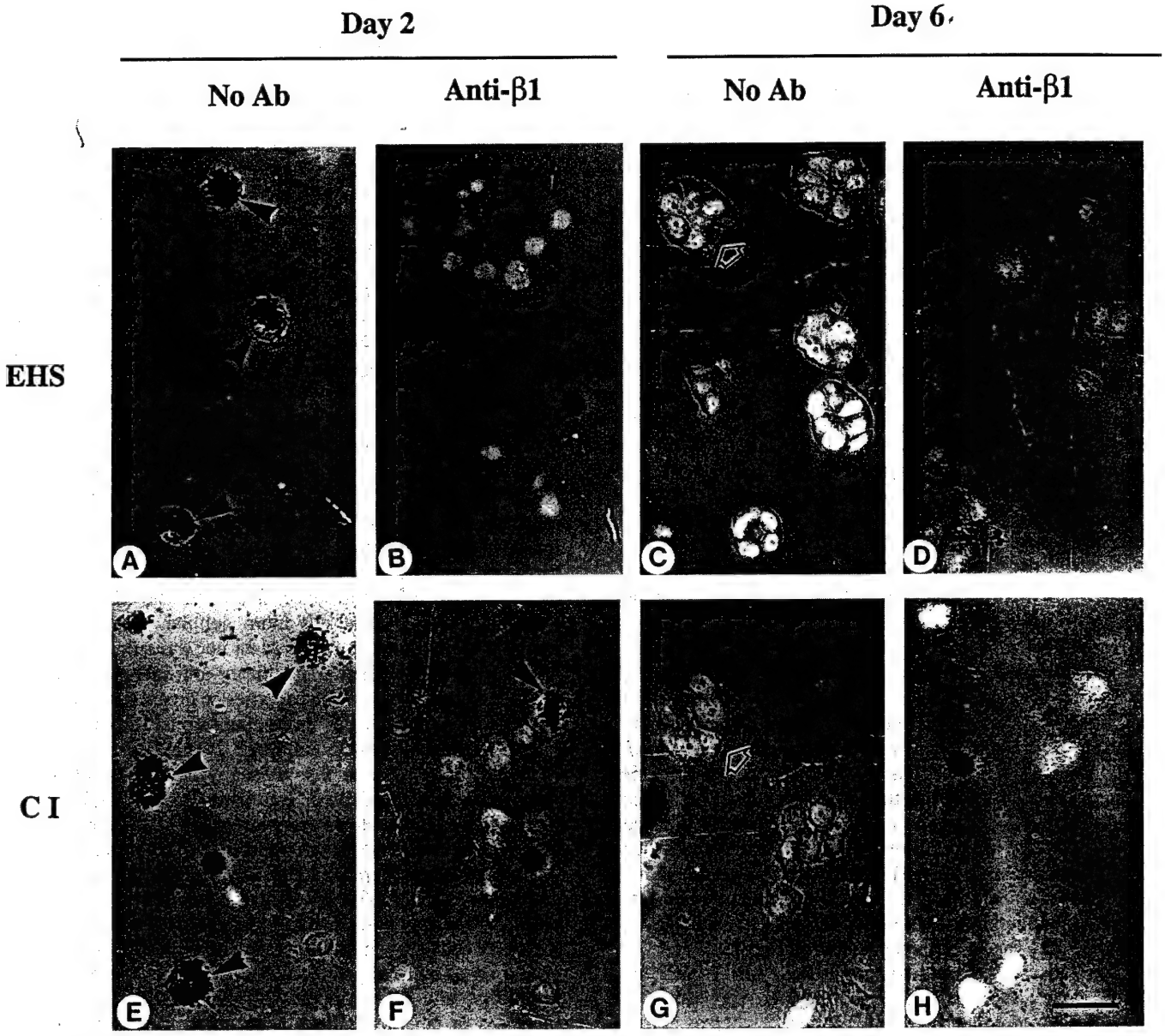

Fig. 4. Inhibition of cellular growth within ECM substrata by function-blocking anti-integrin antibodies. Normal HMT-3522 cells were cultured within EHS matrix (EHS; A-D) and collagen I (CI; E-H) for 2-6 days. Cultures were labeled with [^{3}H]thymidine on day 2 and day 6 of culture. 5 μm frozen sections were stained with DAPI to visualize nuclei and processed for autoradiography. At day 2 a high proportion of nuclei are labeled with thymidine (arrowheads) in both ECM substrata in the absence of inhibitory anti-integrin antibodies (A,E). In contrast, few nuclei are labeled at day 2 in the presence of 100 μg/ml inhibitory anti-β1 antibody (clone AIIB2) (B,F). By day 6 HMT-3522 cells cultured without antibodies proceed to form growth-arrested acinar structures in both ECM substrata (C,G) (open arrows), whereas in the presence of inhibitory anti-β1 antibodies the cells do not incorporate thymidine and fail to form acinar structures (D,H). Bar, 33 μm.

and MCF-7/9 cells. The percentages of nuclei stained with ApopTag reagents for these three carcinoma lines were 4.85%, 0%, and <1%, respectively, without antibodies and 5.6%, <1% and <1% with inhibitory antibodies. These data demonstrate that, even though certain integrin subunits are expressed in these carcinoma cell lines (Table 1, Fig. 1), interfering with their function does not block cell growth, inhibit colony formation or induce apoptosis in these cells (Fig. 6).

DISCUSSION

eractions between mammary epithelium and extracellular matrix have been shown to be critical to mammary-specific growth and differentiation. Mouse and human mammary epithelial cells cultured on, or within, reconstituted basement membrane undergo a process of morphogenesis to form polarized acinar structures that secrete sialomucins and milk proteins apically and deposit basement membrane components basally. Tumor cells, on the other hand, fail to recapitulate this process and produce large, poorly differentiated colonies. These data suggest that interactions between mammary epithelial cells and their microenvironment are important in regulating normal cellular functions and that these interactions are disturbed in tumorigenesis.

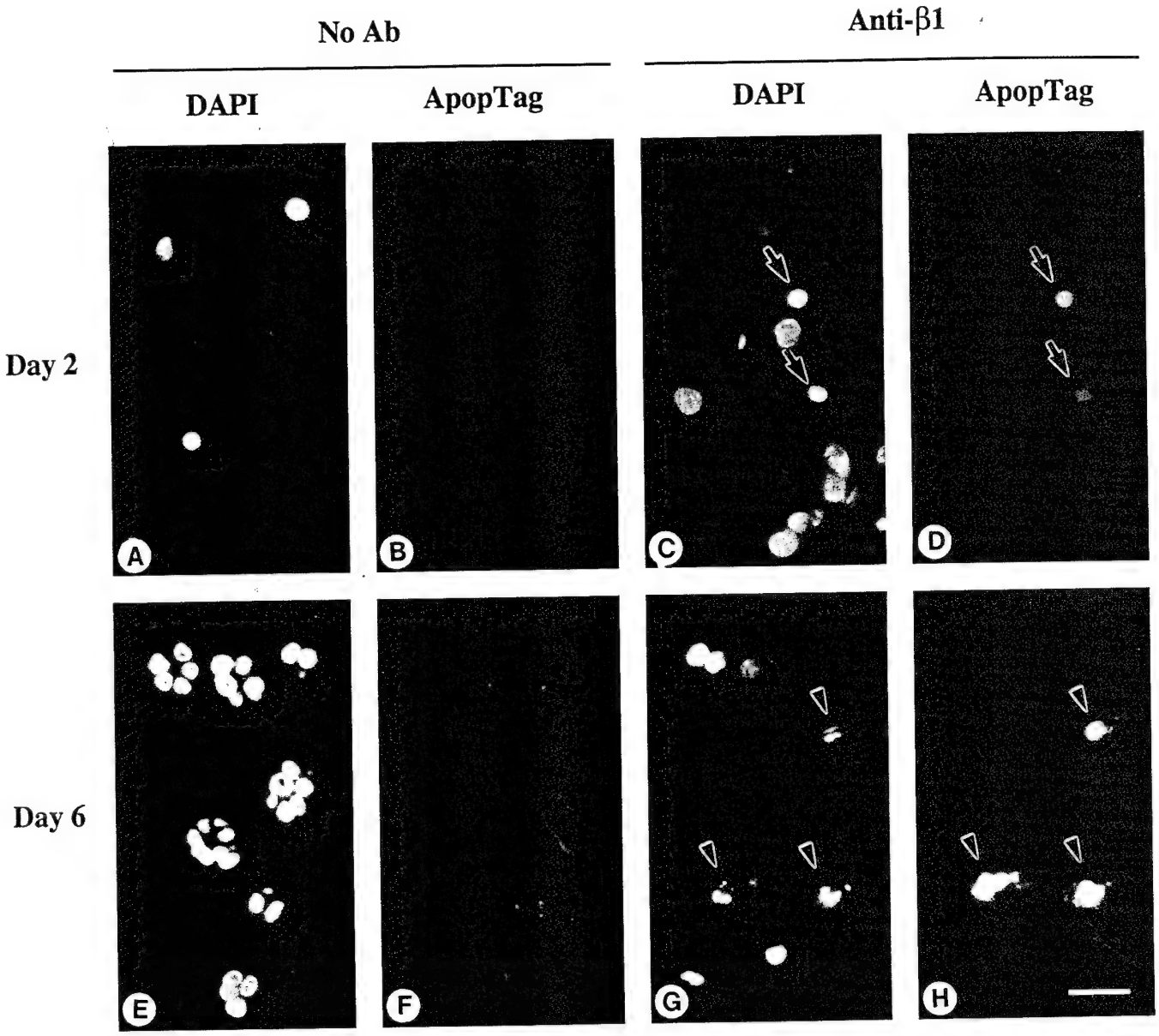

Fig. 5. Induction of apoptosis within EHS matrix by function-blocking anti-integrin antibodies. Normal HMT-3522 cells were cultured within EHS matrix for 2-6 days. 5 μm frozen sections were double-stained with DAPI to visualize nuclei (A,C,E,G) and with FITC-conjugated anti-digoxigenin to detect digoxigenin-labeled genomic DNA in apoptotic nuclei (B,D,F,H). (A-D) 2-day cultures of HMT-3522 cells suspended in EHS matrix without antibodies (A,B) or with 100 μg/ml inhibitory anti-β1 integrin antibodies (clone AIIB2) (C,D). (E-H) 6-day cultures of HMT-3522 cells without antibodies (E,F) or with inhibitory anti-β1 integrin antibodies (G,H). Note the presence of apoptotic nuclei (arrowheads) in cells which remain suspended as single cells after 6 days of culture with anti-β1 integrin antibodies (H). Bar, 25 μm.

Interactions with ECM are mediated by integrins, and there is evidence in mouse cells that β1 integrin signaling is involved in mammary epithelial differentiation in culture in response to basement membrane (Streuli et al., 1991). The basement membrane response is now known to be mediated by laminin (Streuli et al., 1995).

Human mammary epithelial cells in vivo express several integrins that recognize laminin. The expression of these integrins has been found altered in the majority of human breast carcinomas studied and the severity of receptor losses or downregulation correlates with tumor grade (Pignatelli et al., 1991; Koukoulis et al., 1991, 1993). This suggests that dysregulation of integrin expression may be an important parameter in breast tumorigenesis. Our analysis of the profile of integrin subunit expression in normal HMT-3522 cells and three breast carcinoma cell lines, HMT-3909/S13, MCF-7/9 and MDA-MB-435, following culture in reconstituted basement membrane, broadly agrees with published in vivo observations. Normal HMT-3522 cells showed weak expression of α1 in the cytoplasm and α2 at the basolateral surface, and abundant expression of α3 and β1, primarily at the basolateral surface, and α6 and β4 at the basal cell surface. These data suggest that α2β1, α3β1, α6β4 and potentially α6β1 are the major integrins for laminin expressed in HMT-3522 cells. The significance of the α1 integrin staining in the HMT-3522 cells is not clear as previous reports have dem-

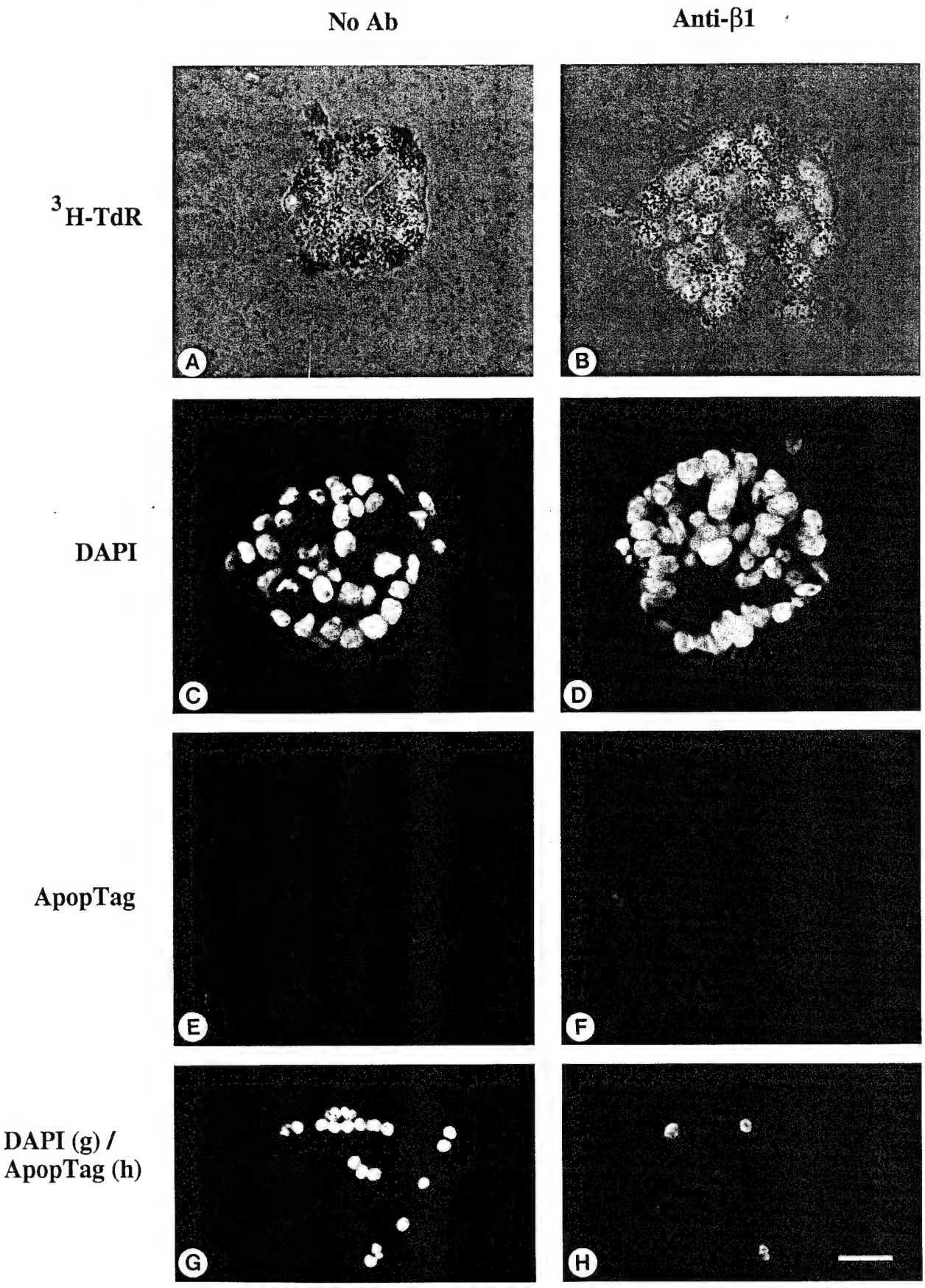
No Ab
Anti-β1
^{3}H-TdR
DAPI
ApopTag
DAPI (g) /
ApopTag (h)
A
B
C
D
E
F
G
H

Fig. 6. Effect of inhibitory anti-integrin antibodies on tumor colony formation, cellular growth and cell survival. MCF-7/9 cells were cultured for 6 days in reconstituted BM with no antibodies (A,C,E,) or with 100 μg/ml inhibitory anti-β1 integrin antibodies (clone AIIB2) (B,D,F). Cultures were labeled with [^{3}H]thymidine and 5 μm frozen sections were stained with DAPI to visualize nuclei and then either processed for autoradiography (A,B) or stained with FITC-conjugated anti-digoxigenin to detect digoxigenin-labeled genomic DNA in apoptotic nuclei (B,D,F). (A,C,E) Colonies formed by MCF-7/9 cells cultured without antibodies that have thymidine-labeled nuclei (A) but no apoptotic nuclei (C, DAPI; E, FITC-anti-digoxygenin). (B,D,F) Similar colony formation by MCF-7/9 cells cultured with anti-β1 integrin. The colonies also have thymidine-labeled nuclei (B) but no apoptotic nuclei (D, DAPI; F, FITC-anti-digoxygenin). (G,H) Control human peripheral blood leukocytes stained with DAPI (G) and FITC-anti-digoxygenin (H). Note the presence of apoptotic nuclei in the leukocytes (H). Bar, 25 μm.

strated α1 localization in both luminal epithelial (Koukoulis et al., 1993) and myoepithelial cells of the breast (Lazard et al., 1993; O. W. Petersen, unpublished observations). The breast carcinoma cell lines HMT-3909/S13, MCF-7/9 and MDA-MB-435 showed loss, disordered expression, or downregulation of these integrin subunits. The changes were most severe in the metastatic MDA-MB-435 cells, suggesting a correlation between dysregulation of integrin expression and aggressive tumor behavior consistent with the in vivo survey data discussed above. These data validate our reconstituted basement membrane assay, provide new markers of differentiation to distinguish normal and malignant breast epithelial cells in our 3-D assay, and provide a basis for evaluating the functional roles of integrins in mediating signals from ECM for mammary-specific differentiation in culture.

The formation of polarized acinus-like spheres is one parameter of normal mammary function that is regulated by basement membrane. This morphogenesis has been shown in turn to be required for biosynthetic aspects of mammary differentiation, including the expression of the milk whey acidic protein in mouse cells (Chen and Bissell, 1989; Lin and Bissell, 1993), apical secretion of sialomucin, and formation of endogenous basement membrane in human breast cells (Petersen et al., 1992; Howlett et al., 1994). Through the use of specific inhibitory anti-integrin antibodies, we have shown that the morphogenesis of acinar structures by normal breast epithelial cells is dependent on integrins. Inhibitory antibodies specific for the β1 integrin subunit virtually abolished the sphere-forming capacity of normal HMT-3522 cells in both reconstituted basement membrane and in type I collagen gels. Preliminary data with another normal mammary epithelial cell line MCF-10A (Soule et al., 1990) showed similar trends (data not shown). These data suggest that integrins are involved in a general mechanism by which mammary epithelial cells perceive morphogenetic signals from the ECM. Our experiments with inhibitory anti-α subunit antibodies suggest a specificity of integrin signaling that is dependent on the nature of the ECM presented to the cells. The α2β1 receptor may be preferentially utilized for interactions with collagen which is consistent with data showing that α2β1 mediates the morphogenesis of MTSV1-7 breast epithelial cells (Berdichevsky et al., 1992, 1994; D'Souza et al., 1993) and the well differentiated breast carcinoma cell line T47D (Keely et al., 1995) induced by type 1 collagen. In contrast, α3β1 may be preferred for interactions with basement membrane. Both receptors recognize laminin and collagen (Hynes, 1992). Thus, the lack of inhibition of HMT-3522 morphogenesis by anti-α2 antibody in EHS and anti-α3 antibody in collagen suggests that mammary cells can modulate either the expression of these receptors or their binding affinities depending on the ECM ligands encountered. Support for the latter possibility was provided by observations on MTSV1-7 cells transformed by the c-*erb*-B2 gene in which losses of α2β1, but not α3β1, lead to a failure to undergo morphogenesis in collagen gels (D'Souza et al., 1993). This suggests that although MTSV1-7 cells have α3β1 integrin, they do not use this receptor for formation of 3-dimensional structures in collagen. The lack of inhibitory effects of the anti-α6 antibody GoH3 on sphere formation is consistent with the lack of inhibition of basement membrane-induced β-casein expression in mouse mammary cells using this antibody (Streuli et al., 1991) and suggests that α6β1 may not be critical to mammary differentiation in response to BM. However, α6β1 integrin may be important in other tissues such as the lung, since the E8 fragment of laminin which binds to α6β1 (Sonnenberg et al., 1990), is involved in signaling lung alveolar morphogenesis by basement membrane (Matter and Laurie, 1994). The α3β1 integrin binds the E3 fragment of laminin (Gehlsen et al., 1992), rather than E8 and it was recently shown that a domain within E3 is required for the induction of β-casein in mouse mammary cells (Streuli et al., 1995). Although direct evidence for a role for α3β1 in β-casein expression is lacking, these data further suggest a role for α3β1 in basement membrane-induced functions of mammary cells.

The laminin form found in EHS matrix (laminin 1) is known to be a weak ligand for α3β1 integrin, whereas kalinin (laminin 5) shows much stronger affinity for this receptor (Weitzman et al., 1993). Thus, although our data indicate that acinar morphogenesis by mammary cells can be driven by EHS, a role for endogenous BM rich in kalinin or another laminin isoform, cannot be discounted. Our analysis of the capacity of HMT-3522 cells to deposit endogenous BM showed that while both EHS and type 1 collagen can support the formation of spheres, the deposition of endogenous BM was induced in EHS only. These data indicate that endogenous BM is not necessary for sphere formation, but that the level of sphere organization is different in the two substrata. The data also indicate that both type 1 collagen and reconstituted BM can downregulate growth. These data fit with studies on well and poorly differentiated pancreatic carcinoma cell lines cultured in matrigel and type I collagen where endogenous BM deposition was found in matrigel only (Yamanari et al., 1994). While this point is not emphasised, their data also indicate that the formation of 3-D structures in the two substrata is not the same - the cells with a capacity for differentiation are more differentiated in matrigel than in type 1 collagen.

We have observed a growth-inhibitory effect associated with the anti-integrin-mediated block to acinar morphogenesis in EHS matrix and collagen I. Inhibitory anti-β1 integrin antibodies severely inhibited the growth of HMT-3522 cells such that the cells remained in suspension as single cells for the duration of the experiment. Similar results were obtained with MCF-10A cells (data not shown). These data suggest that the initial pulse of cellular growth observed prior to growth arrest

for normal cells in EHS and collagen I (Petersen et al., 1992, and data presented herein) is a prerequisite for acinar morphogenesis by mammary epithelial cells under our present assay conditions where cells are seeded at low density. Previous reports of continuous propagation of normal human mammary epithelial cells in collagen gels (Yang et al., 1982; Sakthivel et al., 1993) may reflect differences in media composition and/or cell densities within the gels. If the pulse of growth observed in our assay is prevented by ligating ECM receptors with inhibitory anti-β1 integrin antibodies, the cells undergo apoptosis. These data support the hypothesis that if integrin-mediated contact with ECM is denied, the cells can neither grow nor differentiate and are unable to survive (Frisch and Francis, 1994; Boudreau et al., 1995; for review see Ruoslahti and Reed, 1994).

An important characteristic of tumors, in addition to loss of growth control, is disrupted tissue histoarchitecture and changes in adhesive cellular interactions as well as cell-ECM interactions. The inability of tumors to produce organized three-dimensional structures in culture has been observed both in reconstituted BM (Petersen et al., 1992,) and collagen gels (Shearer et al., 1992). Most significantly, our data with inhibitory antibodies show that formation of colonies by three breast carcinoma cell lines (HMT-3909/S13, MCF7/9 and MDA-MB-435) proceeds in the presence of inhibitory anti-integrin antibodies. That the antibodies did not inhibit carcinoma cell growth or induce apoptosis suggests that tumor colony formation occurs independently of the integrin signaling utilized by normal cells to produce acinar structures in reconstituted basement membrane. The variable, disordered expression of integrin subunits on the carcinoma cells suggest that those integrins that are present are either insufficient in quantity, improperly localized to deliver a signal to the cell, or otherwise inactive. However, it is highly unlikely that β1 integrins are entirely nonfunctional in these cells, since our previous studies indicate that in the absence of serum, all three of the tumor cell lines required type 1 collagen to adhere and to grow in a 2-dimensional monolayer culture.

Taken together, our data demonstrate that normal breast epithelial cells form acinus-like structures from single cells by a process that involves at least two distinct phases: (1) growth; and (2) conversion of the cell group into differentiated structures. The growth phase is β1-integrin-dependent; if the cells are deprived of the BM signal, the cells enter an apoptotic pathway. In contrast, breast carcinoma cells form disordered colonies independently of β1 integrins; cellular growth is not inhibited and apoptosis is not induced by antibody blockade. Thus, defects in cellular responses to microenvironment, including ECM, may be an important parameter of malignancy in addition to other established genetic lesions. Consistent with the above is the observation that elevation of the expression of the α5β1 receptor for fibronectin by transfection into tumorigenic Chinese hamster ovary cells leads to increases in fibronectin deposition, restoration of anchorage dependence and inhibition of tumorigenicity (Giancotti and Ruoslahti, 1990).

Our data demonstrate that integrins play an active role in both the growth and survival of normal human breast cells. We believe this is also the the first demonstration that tumor cells do not use β1-containing integrins for either of these processes. The mechanisms by which breast carcinoma cells escape integrin-mediated regulation of growth and apoptosis remain to be determined and elucidation of this process will be a valuable step toward further understanding the nature of human breast malignancy.

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The development of a functionally relevant cell culture model of progressive human breast cancer

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Normal mammary homeostasis, and by implication tumorigenesis, are dependent upon the dynamic interplay between epithelial cells, stromal components and the extracellular matrix. To study the evolution of human breast cancer, a functionally relevant cell culture model is required which recognizes the complexity of the mammary gland's microenvironment. The development of an appropriate breast epithelial cancer cell model will be dependent on the ability to recreate the 'normal' and 'neoplastic' tissue microenvironment in culture. Towards this goal, a 3-dimensional extracellular matrix (ECM) assay, employing a reconstituted basement membrane, has been developed which allows for the rapid and accurate discrimination of normal and neoplastic cells when cultured. To investigate stromal/epithelial cell interactions, we have developed a tumor environment assay which essentially mirrors the tumor microenvironment histologically. The use of a novel, near diploid, human breast epithelial cell line, HMT-3522, which has transformed spontaneously with passage in culture, together with these 3-dimensional culture assays is expected to provide meaningful markers of initiation and progression.

Key words: mammary gland / tumorigenesis / ECM / human breast cancer / stroma

HUMAN BREAST CANCER is thought to derive from the stepwise transformation of the luminal epithelial cells of the ducts and terminal lobular units.[1,2] This implies that mutational events are critical to the genesis of the transformed phenotype. Consistent with this prediction, a number of specific genetic lesions have been found in tumor biopsy specimens and tumor-derived cell lines. These mutations include the amplification or aberrant expression of the proto-oncogenes: c-*myc*, c-*erbB2*, *int-2*/*hst-1* and infrequently H-*ras*, as well as, inactivation or deletion of tumor suppressor genes such as: *p53*, *Rb-1*, and *BRCA1* (see chapters by E.Y.H.P. Lee, R. Lupu *et al*, and V. Band, this issue).[3] Despite these clinical correlations, at present it is not yet possible to ascribe a pivotal role for a specific genetic mutation in breast cancer aetiology. To clarify this issue it would be desirable to follow the progressive mutational changes until malignancy ensues in the tissue *in vivo*. However, the time-course of breast tumor evolution can be quite long, taking anywhere from 5–30 years to develop. To follow the development of carcinoma *in situ* to invasive carcinoma, epidemiological protocols must span periods of 10–15 years.[4] This precludes the feasibility of conducting meaningful and reproducible human studies and emphasizes the need to develop appropriate experimental models of human breast cancer progression in culture.

It is also becoming apparent that the activation of a specific oncogene(s) or the loss of tumor suppressor gene function, by themselves, are insufficient to explain the onset of tumor formation. Transgenic mouse models overexpressing MMTV/c-*myc* and v-Ha-*ras* only rarely develop mammary malignancies. Indeed, transplantation of H-*ras* and c-*myc*-transfected mouse mammary epithelial cells into cleared fat pads, will only give rise to carcinomas sporadically.[5,6] That other factors are involved in cancer progression is also exemplified by the example of fibrocystic breast disease. Many breast cancers arise without prior detection of fibrosis, while the existence of fibrocystic breast disease does not necessarily dictate the development of invasive carcinoma.[7] Furthermore, invasive carcinoma can display great behavioral disparity between patients, metastasizing rapidly in one, or demonstrating a latent period of up to 30 years before metastases appear in another.[8] These observations underscore the dynamic and progressive nature of this disease, and serve to emphasize the need to recognize its inherent complexity when attempting to design model systems to study breast cancer. In

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addition, they imply that other factors are also essential for the pathogenesis of mammary neoplasia.

The stroma of the mammary gland accounts for more than 80% of the resting breast volume.[9] This stroma consists of fibroblasts, blood vessels and a macromolecular network composed of glycoproteins and proteoglycans, known collectively as the ECM. It has been demonstrated that this tissue microenvironment plays a fundamental role in both the control of luminal epithelial cell gene expression, and the induction and maintenance of their tissue-specific function. Studies using rodent models have revealed the importance of a reconstituted basement membrane to the morphogenesis and differentiation of mammary luminal epithelial cells.[10] When placed upon a reconstituted-basement membrane gel, mammary epithelial cells undergo morphogenesis to form spheroids that exhibit many of the structural and functional characteristics of alveoli *in vivo*.[11,12] ECM also actively directs tissue-specific gene expression. Expression of the abundant milk protein beta-casein has been shown to be regulated transcriptionally by an ECM response element located upstream of the gene.[13] The dynamic role of this microenvironment in cellular homeostasis is also underscored during neoplasia. Perturbations in the production, deposition and degradation of the ECM are characteristically observed in rodent mammary carcinomas both *in vivo* and in culture (A. Lochter and M.J. Bissell, this issue). Transgenic mice aberrantly expressing stromolysin-1, an ECM degrading enzyme, were shown to undergo premature involution in pregnancy and excessive branching while in the resting state.[14] Continued studies with these mice has recently demonstrated that they develop mammary tumors, sometimes as early as 4 months after birth (see chapter by C. Sympson *et al*, this issue). These data suggest that perturbation of tissue microenvironment by metalloproteinases is sufficient to lead to tumor formation. Similarly, human breast carcinomas are associated with aberrant stromal cell expression of ECM-degrading proteinases, while invasive human breast carcinoma is characterized by the rupture of the basement membrane and the infiltration of the surrounding stroma.[15,16] Indeed, a profound stromal response (desmoplasia) consisting of changes in cellular composition and extracellular components, is commonly observed in human infiltrating ductal carcinomas.[17] Human adenocarcinoma is typified by the proliferation of fibroblasts, the appearance of myofibroblast-like cells expressing alpha-smooth muscle actin and extreme changes in the synthesis and deposition of collagen I and III.[18,19].

There are tissue differences between rodents and humans which need to be addressed. In contrast to the mouse, human mammary epithelial cells are not embedded directly in fat tissue. Instead, they are surrounded by a separate intralobular stroma, consisting of collagenous connective tissue and varying amounts of resident fibroblasts lying as scattered single cells. The adipocyte and epithelial compartments, in turn, are separated and surrounded by a dense interstitial stroma, which is inhabited by blood vessels (Figure 1).[19] In addition to species differences in normal tissue architecture, the breast cancers produced in rodents are not morphologically identical, nor do they invade and metastasize the same as human breast cancers.[20] Therefore, a dissection of the functional significance of these interactions in the genesis of human breast cancer will, in our opinion, benefit from the design of novel culture systems for human cells to address these questions.

Ideally a human breast cancer model should be specific enough to resolve, within a realistic timeframe, the subtle phenotypic conversions observed during human carcinogenesis *in vivo*. Furthermore, the assay should take into account the complex interplay between cell types and the tissue microenvironment. Finally it should allow for easy experimental manipulation of the system, to alter genetic information and cell–cell interactions in the context of a changing microenvironment. Needless to say, this type of model system has not been readily forthcoming. Nevertheless, in recent years there has been much progress towards attaining these goals. In this short review, we briefly discuss the human cell cultures presently available for the study of human breast cancer. The three-dimensional model systems we discuss have recently been developed in our laboratories.

Spontaneously derived mammary epithelial cell lines

Most human breast cell lines are derived from spontaneously immortalized cells with a pre-existing immortal phenotype, and hence yield little insight into the evolution of breast cancer from early benign lesions and normal breast epitheliel cells.[21-23] Indeed, cellular immortalization is not a necessary prerequisite of the tumor phenotype.[24] All established tumor cell lines analysed are aneuploid, often as the result of

genetic instability and thus display heterogeneous phenotypes (see ATCC catalogue of cell lines). While this may represent a drawback in some experiments, established cell lines have been particularly valuable in experiments designed to address the role of mutant oncogenes and tumor suppressor genes in basic cellular processes. For example, retroviral-mediated gene transfer of wild-type *p53* and Rb into the mammary tumor cell lines, MDA-MB468 and BT549 respectively, led to the reduction of their ability to grow in soft agar and partially suppressed MDA-MB468 tumor formation in nude mice. Since MDA-MB468 and BT549 contain a partially deleted Rb gene, as well as the point-mutated *p53*, these data demonstrate the dominant tumor suppressing capabilities of these alleles (see chapter by E.Y.H.P. Lee, this issue).[25] Nevertheless, the usefulness of these cell lines as models of early breast cancer may be limited. Thus, caution should be exercised when extrapolating results obtained with these cell lines to neoplasia *in vivo*.

A few spontaneously immortalized non-malignant human mammary epithelial cell lines exist, which can be used to answer questions about normal breast epithelial cell biology. These include: MCF10A and HMT-3522, both established from women with fibrocystic disease, HBL-100, which has been shown to harbor the SV40 virus, and a cell line newly

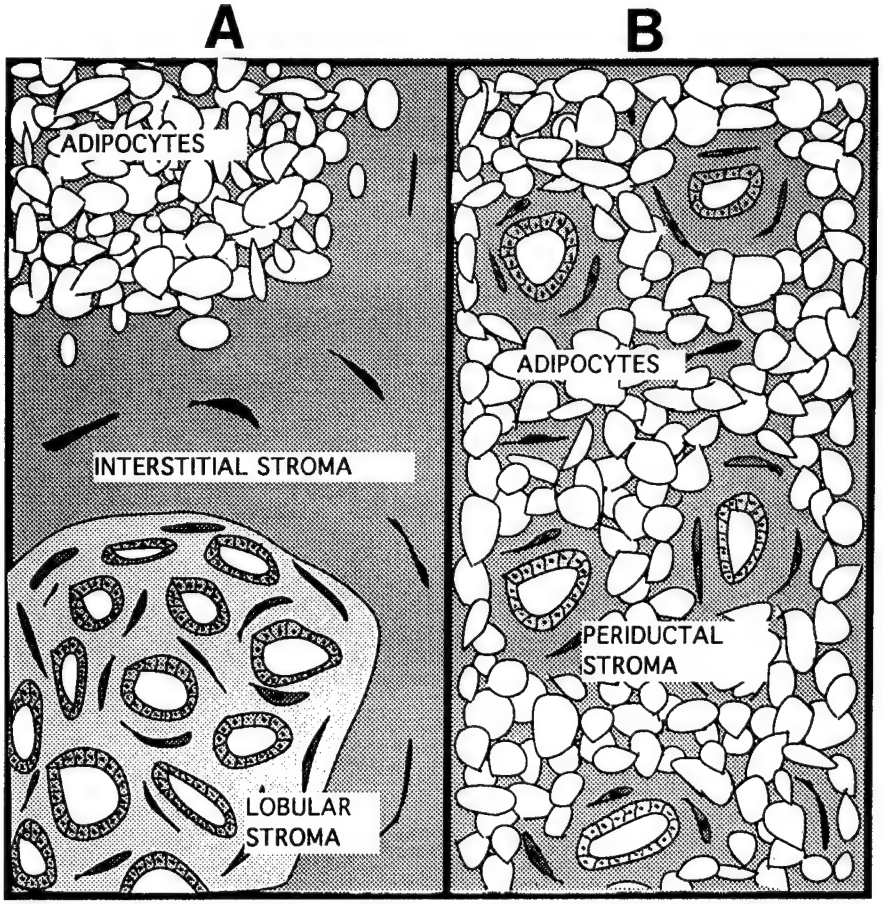


Figure 1. Diagramatic comparison of human (Panel A) and mouse (Panel B) mammary gland anatomy. The epithelial compartment of the human breast is surrounded by a basement membrane, which is embedded in an intralobular stroma of extracellular matrix and varying amounts of resident fibroblasts. The adipocyte and epithelial compartments, in turn, are separated and surrounded by a dense interstitial stroma, which is inhabited by blood vessels. The epithelial compartment of the mouse breast is surrounded by a basement membrane, and embedded directly in a periductal stroma consisting of resident fibroblasts, blood vessels and adipocytes. (Adapted and reproduced with permission.)[19]

established by the spontaneous in culture immortalization of human breast epithelial cells from a patient with Li Fraumeni syndrome.[26, 29] At present, however, their usefulness for studying breast cancer progression remains uncertain. In this respect, efforts have been directed at establishing a correlative tumorigenic line. MCF10Aneo T was derived following transfection of MCF10A with the mutated T-24 H-*ras* gene and displays a transformed phenotype in culture.[30] Long-term tumorigenic potential of MCF10Aneo T was demonstrated by a xenograft technique. Alternation of dorsal flank cell transplantation, with tissue culture passage, eventually gave rise to atypical hyperplasia and carcinoma *in situ*, in nude/beige mice.[31] However, the need for complex manipulations, and the feasibility of reproducibility, raise questions about the usefulness of this putative model.

Primary cultures of non-malignant and malignant mammary epithelial cells

Primary cultures of normal human mammary epithelial cells have been established from cells isolated from surgical discard tissue (reduction mammoplasty), and from milk exudates (see chapter by V. Band, this issue).[32,33] This has been achieved by using a combination of specially defined media and rigorous isolation protocols.[34,35] As such, these cultures have been instrumental in furthering our understanding of normal human mammary cell physiology. A caveat of freshly prepared primary cultures however, is their finite cell passage potential (3–5 passages) and the labor intensive protocols involved in generating these cultures. This, in turn, limits the type and design of feasible experiments. In an effort to circumvent these problems, pure epithelial clumps (organoids), isolated from reduction mammoplastics, have been kept frozen as a readily available stock of primary culture material. Also, media have been derived that allow the self-selected expansion of these primary cells, to generate clones capable of sustaining 7–24 passages.[36] Alternately, self-selected clonal variants, of primary breast cultures have been chemically transformed by mutagenesis with benzo(a)pyrene, to generate two immortalized cell lines denoted 184A1 and 184B5.[37] Similarly, mature breast epithelial cells, isolated from milk exudates, have been rendered immortal by viral transfection with the SV40 T antigen.[38] One drawback of these methods is the resultant genotypic and phenotypic changes induced by these manipulations. For example, it has been demonstrated that SV40 T antigen transfection inactivates both *p53* and Rb.[39]

In contrast to normal breast cell strains, cultures of primary breast carcinomas have been extremely difficult to establish (see chapter V. Band, this issue).[19] To develop breast cell models to study carcinogenesis, researchers have resorted to physical and chemical manipulations. Complete malignant transformation of a normal human mammary epithelial cell strain, 76N, was observed following fractionated gamma irradiation.[40] The lack of knowledge as to the precise nature of the mutational events induced, and their rarity so far have precluded the routine experimental usefulness of this method. Transfection and viral infection of the cell lines 184A1 and 184B5 with the mutated oncogenes Ki-*ras* or *erbB2*, or a combination of the SV40-T and mutant Ha-*ras* oncogene has successfully achieved complete transformation of these cells.[36,41] Similarly immortalization and partial transformation has also been observed following transfection of human milk exudate and breast tissue cells with the herpes papilloma viral oncogenes E6 and E7 (see chapter by V. Band, this issue). Thus, infection with E6 and E7 retroviruses together induced complete immortalization and anchorage-independent growth of milk-derived epithelial cells. These transformed cells fail to produce tumors in nude mice, and therefore probably represent a preneoplastic transformation phenotype. Full tumorigenic transformation was induced by transfection with a second oncogene, the mutated H-*ras* (see chapter by V. Band, this issue). While these engineered cell lines have been invaluable tools for many biochemical and functional studies, it is important to realize that most of the oncogenes used to confer complete malignant transformation are not known to be associated with human breast cancer. Therefore, the comparisons that have been made between cultured non-malignant and malignant transformed human cells may not accurately reflect neoplasia *in vivo*. Nevertheless, a combination of all of these cell lines, incorporating a careful examination of their phenotypes, should enable us to arrive at a more complete comprehension of key neoplastic events.

The importance of cellular environment

A caveat in traditional monolayer culture is the nonphysiological two-dimensional organization, excessive cell growth-rates similar to malignant cells,

and the lack of distinguishable neoplastic phenotypes.[42] Despite these drawbacks, most of the work conducted thus far on human primary mammary cells and cell lines has been on two-dimensional substrata.

Studies using a 3-dimensional basement membrane gel culture system in rodents, have elucidated mammary-specific responses to ECM at the morphogenetic, cellular and molecular levels. The same rationale have now been applied to normal and neoplastic human mammary epithelial cell lines and primary cultures. Defined cell–ECM interactions can be exploited rapidly and accurately to distinguish normal and malignant breast cells in culture.[43] Using this assay system, we demonstrated the capacity of normal human breast epithelial cells to re-express their in-vivo patterns of growth and differentiation. Cells from reduction mammoplasties and non-malignant cell lines including HMT-3522 and MCF10A formed polarized organotypic spheres resembling acini *in situ*. These cells also deposited an endogenous basement membrane and subsequently growth arrested after 7–10 days of culture (Figure 2A and inset). In contrast, primary breast carcinoma and tumorigenic breast cell lines formed large, dense and unpolarized colonies of cells and failed to deposit basement membrane or growth arrest (Figure 2B and inset). Subsequently, similar differences between normal and malignant primary cells were observed by Bergstraesser and Weitzman (1993), when cultured on basement membrane gels.[44] Three-dimensional basement membrane cultures have also been used to define the morphogenic behavior of SV40-immortalized and transformed human mammary cells.[45] Based on these differences, we hypothesized that the ability to sense the basement membrane correctly could be the function of a new class of tumor suppressor genes.[43] Indeed, this culture system was recently used to uncover a latent function for the putative metastasis suppressor gene nm-23. Altered expression of nm23 is associated with poor clinical prognosis and aggressive breast cancer behavior *in vivo*.[46] Overexpression of nm23 in the human mammary MDA MB 435 breast carcinoma cell line, was shown to reduce the metastatic potential of the parental cell line in nude mice.[47] When cultured using the 3-dimensional assay system, the parental MDA MB 435 cells demonstrated characteristic malignant behavior whereas the nm23 overexpressing cells were similar to early passage HMT-3522 cells (Figure 3).[48] The normal behavior of these cells were further demonstrated by growth kinetics, as well as by morphological and immunochemical examination.[43,48]

A culture model of spontaneous breast cancer progression

As mentioned previously, HMT-3522 cells were derived from a woman with fibrocystic disease.[27] It was later hypothesized that the immortalization of these cells even if from benign origin, constituted the initial step in malignant transformation.[49] Therefore clonal selection, with propagation of these cells in culture, would eventually give rise to neoplastic changes. Consistent with this theory, preliminary data show that indeed continued passage and selection of these cells in chemically defined media, for more than 300 passages, resulted in spontaneous malignant transformation.[50] Despite these pathological changes, the karyotype of these tumor cells along the continuum, remained remarkably stable.[49,51] This cell line then, comprises the first culture model of spontaneous breast cancer progression.

The role of the microenvironment in mammary neoplasia: critical questions

There are a number of critical questions that need to be addressed regarding the role of the microenvironment in breast cancer aetiology. Primary among these issues is comprehending the complex interplay between stromal components/breast epithelial cells and the ECM in normal and malignant mammary tissue homeostasis. This could be achieved by developing three-dimensional ECM systems employing two or more mammary cell types and studying their interactions. To this end, breast tumor histology has recently been successfully recapitulated in culture, using a newly developed tumor environment assay (Figure 4a-f). This assay system was used to determine the origin of the cells in the stromal reaction; a process that occurs during tumorigenesis *in vivo*.[18,19]

Another important challenge for breast cancer research is the homing of primary tumor cells to their metastatic tissue sites. What tissue factors determine the choice of metastatic tumor site? What role, if any, does the stromal environment of the metastatic tissue site play in determining successful tumor growth? Does it determine the rate or degree of further metastasis? The relevance of this possibility has been underscored by Chung (1993), who studied the

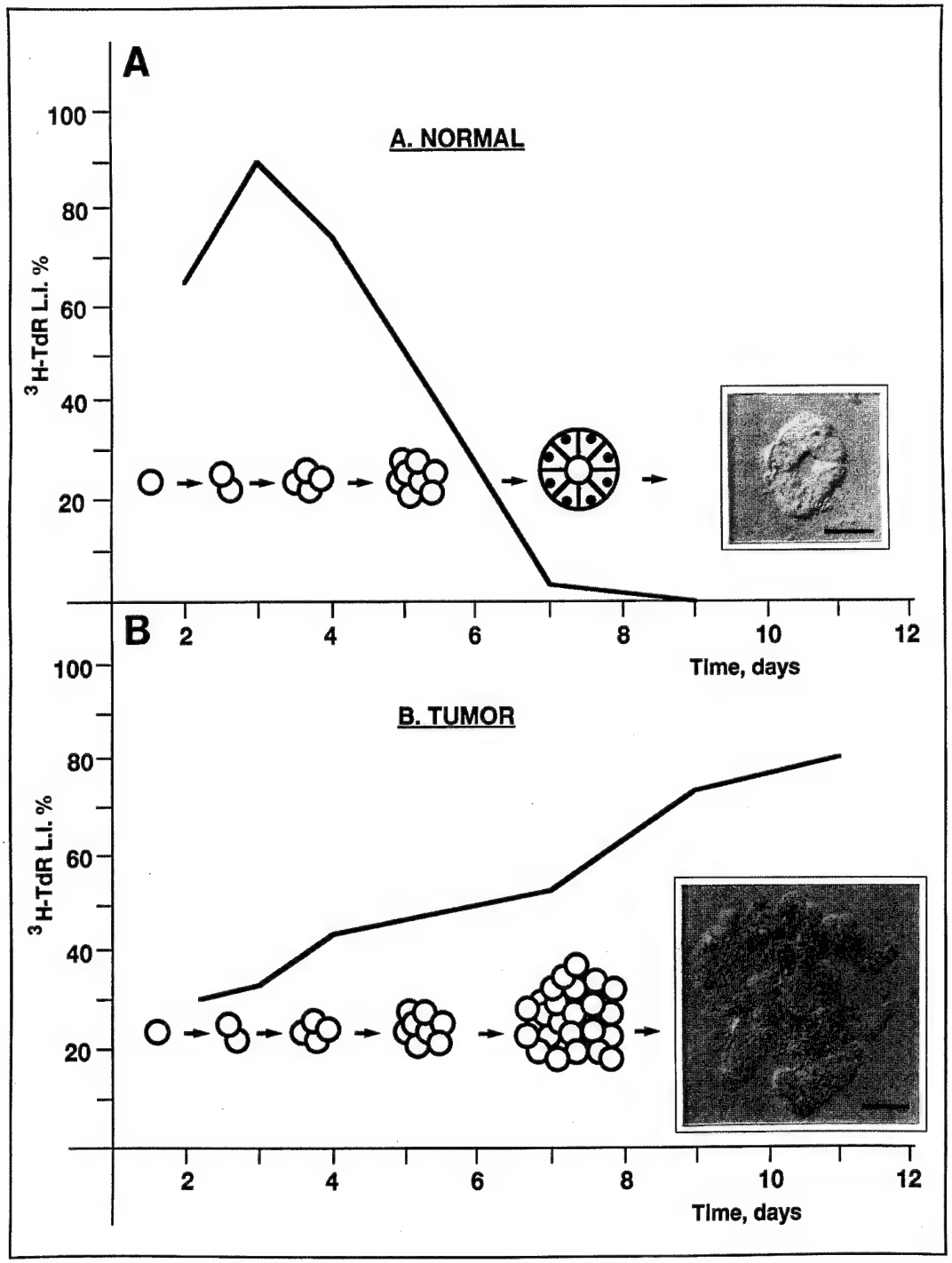
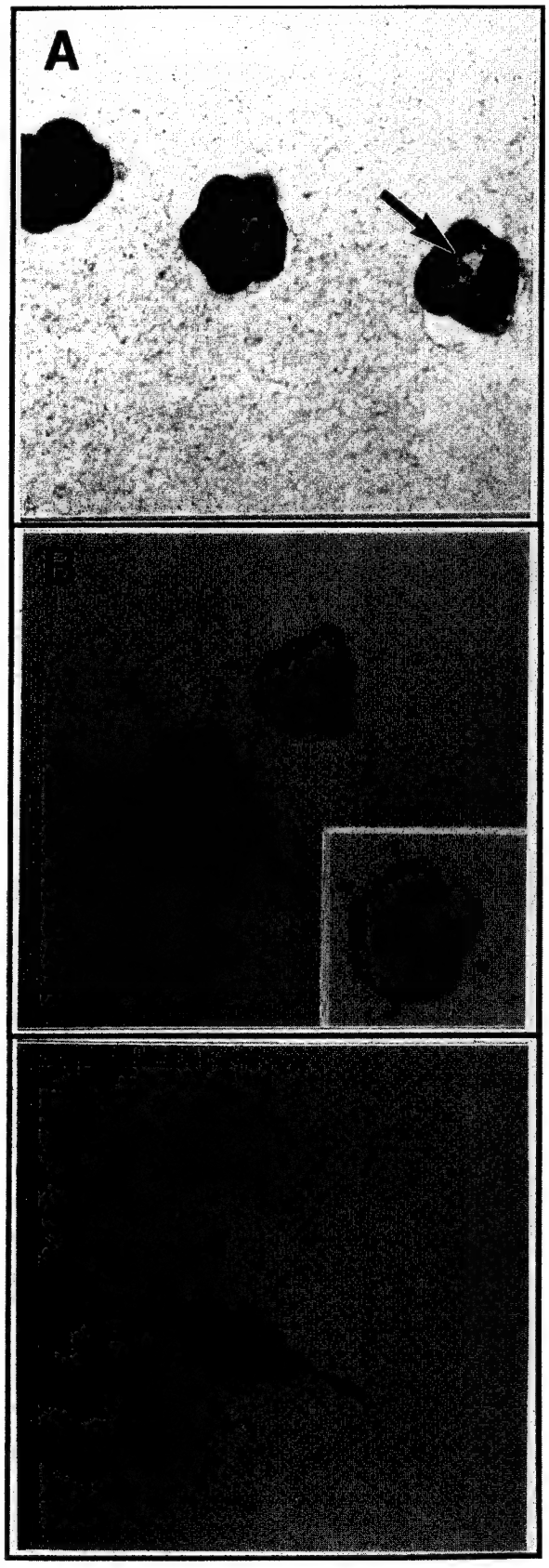


Figure 2. Different growth and morphological responses of early passage, non-malignant mammary epithelial HMT-3522 cells and MCF-7-g carcinoma cells (a subclone of MCF-7 that can grow in the absence of serum) to culture within a laminin-rich basement membrane matrix. HMT-3522 cells growth arrested within 7-10 days of culture in 3-dimensional ECM, illustrated by thymidine labeling indices (Panel A). These cells also formed polarized organotypic spheres resembling acini *in situ*, illustrated by a Namarski image of day 6 thymidine-labelled cryosectioned cultures (Panel A inset). In contrast, MCF-7 (9) cells failed to growth arrest even after 12 days of culture in 3-dimensional ECM (Panel B), and formed large, dense and unpolarized colonies of cells at day 6 of culture (Panel B inset). Bars = 23 μm.



growth enhancing effects of bone stroma on prostatic cancer metastases.[52] For breast cancer this could be examined by co-culturing normal and tumorigenic breast epithelial cells with stromal cells from bone, brain or lung, frequent metastatic sites.[53]

A third consideration for experimental investigation is the role of the mammary adipocyte in breast cancer aetiology. Epidemiological studies have shown an association between a high-fat diet and a high mortality rate from breast cancer.[54] Consistent with these observations, rodent studies have clearly demonstrated a promotional effect of a high fat diet on the frequency of atypical hyperplasia and adenocarcinoma of the breast.[55] Since members of the fatty acid binding protein family appear to be differentiation factors for the mammary gland,[56] the potential interplay between adipocytes and epithelial cells should also be examined, possibly using a similar tumor environment assay as described above. In summary the continued development of 3-dimensional, organotypic tissue culture assays in the context of appropriate temporal models, should provide a fertile area for future research.

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Figure 3. Normal phenotypic behavior of early passage HMT-3522 cells when cultured in a 3-dimensional laminin rich basement membrane matrix for 12 days. Early passage HMT-3522 cells formed polarized organotypic spheres resembling acini *in situ* (Panel A), deposited collagen type IV (Panel B) and laminin around their basolateral membranes (Panel B inset) and exhibited directional secretion of sialomucin (Panel C), as illustrated by hematoxylin and eosin-(Panel A) and immunohistochemical-(Panels B, B inset and C) stained cryosections. Panels A, B and C, × 400; B inset original magnification × 320. (Panels B, B inset and C, adapted and reproduced with permission.)[48]

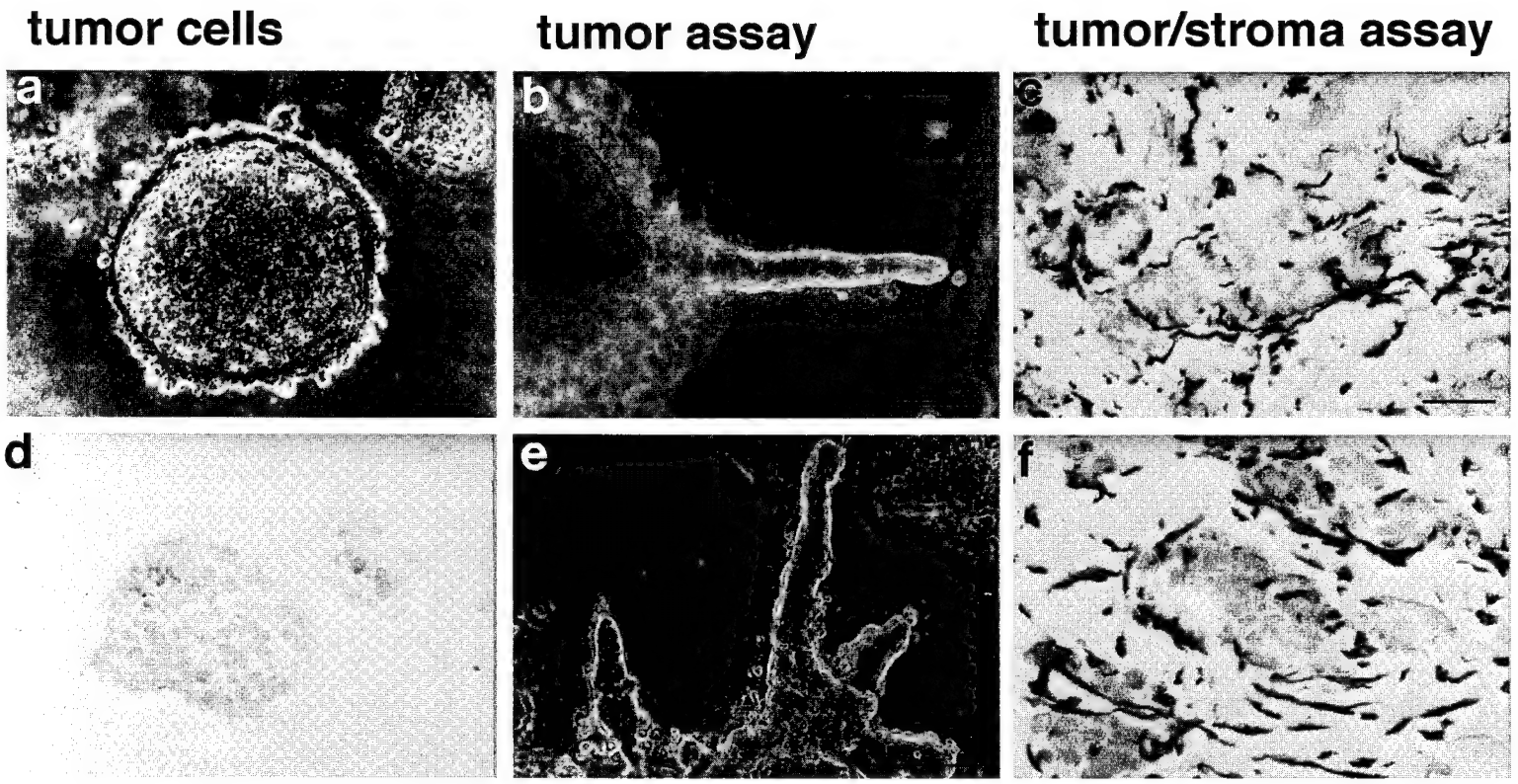

Figure 4. The tumor environment assay: recapitulation of tumor histology when stromal cells are cocultured with tumor cells in three dimension. Tumor cells formed sphericalcolonies (a and b) when cultured alone inside Type 1 collagen gels as demonstrated by phase contrast microscopy (a), and did not express vimentin in immunoperoxidase stained cryosections counter-stained with hematoxylin (b). Co-cultivation with stromal cells in tumor environment assay (c and d) resulted in tumor cell spreading along stromal cells (c), and interaction increased with stromal cell density (d). Immunoperoxidase staining for vimentin and counter-staining of nuclei with hematoxylin (e and f) showed that the typical histology of tumors *in vivo* (e) was virtually reproduced in culture (f). a,b,e, and f, $\times 160$; bar, 50 μm; c $\times 110$; bar, 100 μm d $\times 280$; bar = 50 μm. (Reproduced with permission.)[18]

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A Novel Function for the nm23-H1 Gene: Overexpression in Human Breast Carcinoma Cells Leads to the Formation of Basement Membrane and Growth Arrest

*Anthony R. Howlett, Ole W. Petersen, Patricia S. Steeg, Mina J. Bissell**

Background: **We have developed a culture system using reconstituted basement membrane components in which normal human mammary epithelial cells exhibit several aspects of the development and differentiation process, including formation of acinar-like structures, production and basal deposition of basement membrane components, and production and apical secretion of sialomucins. Cell lines and cultures from human breast carcinomas failed to recapitulate this process. The data indicate the importance of cellular interactions with the basement membrane in the regulation of normal breast differentiation and, potentially, its loss in neoplasia.** ***Purpose:*** **Our purpose was to use this assay to investigate the role of the putative metastasis suppressor gene nm23-H1 in mammary development and differentiation.** ***Methods:*** **The metastatic human breast carcinoma cell line MDA-MB-435, clones transfected with a control pCMVBamneo vector, and clones transfected with pCMVBamneo vector containing nm23-H1 complementary DNA (the latter of which exhibited a substantial reduction in spontaneous metastatic potential in vivo) were cultured within a reconstituted basement membrane. Clones were examined for formation of acinus-like spheres, deposition of basement membrane components, production of sialomucin, polarization, and growth arrest.** ***Results:*** **In contrast to the parental cell line and control transfectants, MDA-MB-435 breast carcinoma cells overexpressing Nm23-H1 protein regained several aspects of the normal phenotype within reconstituted basement membrane. Nm23-H1 protein-positive cells formed organized acinus-like spheres, deposited the basement membrane components type IV collagen and, to some extent, laminin to the outside of the spheres, expressed sialomucin, and growth arrested. Growth arrest of Nm23-H1 protein-positive cells was preceded by and correlated with formation of a basement membrane, suggesting a causal relationship.** ***Conclusion:*** **The data indicate a previously unidentified cause-and-effect relationship between nm23-H1 gene expression and morphological–biosynthetic–growth aspects of breast differentiation in this model system.** ***Implications:*** **While the basement membrane microenvironment is capable of directing the differentiation of normal human breast cells, neoplastic transformation abrogates this relationship, suggesting that intrinsic cellular events are also critical to this process. The data identify nm23-H1 gene expression as one of these events, suggesting an important role in the modulation of cellular responsiveness to the microenvironment. The data also identify previously unknown growth inhibitory effects of nm23-H1 gene overexpression.** **[J Natl Cancer Inst 86:1838-1844, 1994]**

While inappropriate changes in cell growth have been considered the hallmark of cancer, alterations in other characteristics such as cell–cell and cell–extracellular matrix interactions and apoptosis have also been recognized as important determinants of the malignant phenotype. We have recently developed a model system for the morphological and functional differentiation of human breast cells, based on their interaction with a basement membrane-containing microenvironment. When cells from 12 reduction mammoplasties and two normal cell lines were cultured within reconstituted basement membrane (Engelbreth–Holm–Swarm [EHS] matrix), they formed organized acinus-like structures, frequently deposited a basement membrane to the outside of the acinus, secreted sialomucin to the inside of the acinus, and growth arrested (*1*). In contrast, two primary human breast carcinoma cultures as well as six established breast carcinoma cell lines failed to recapitulate this process. Gross distinctions in growth rates and culture morphology were not apparent between normal cells and malignant cell lines on tissue culture plastic. The data indicate dedifferentiation as an important correlate of malignant progression in human breast epithelial cells and confirm the importance of cell–basement membrane interactions in this process (*1*).

Decreased expression of the nm23 family of genes has been associated with histopathologic and/or clinical course correlates

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See "Notes" section following "References."

of aggressive breast carcinoma in several cohort studies (*2-8*). Functional analysis of nm23-H1 gene overexpression was reported in the human MDA-MB-435 breast carcinoma cell line. Both bulk transfectants as well as stable, high expression clones exhibited a significant reduction in metastatic potential in vivo (*9*). In addition, clonal nm23-H1 transfectants exhibited a reduced responsiveness to transforming growth factor-β (TGF-β) in soft agar colonization assays in culture (*9*) and to insulin-like growth factor, platelet-derived growth factor, and serum in motility assays in culture (*10*). The biochemical mechanism of Nm23 protein action is not known; however, the recent identification of an Nm23 serine phosphorylation that correlated with its metastasis-suppressive activity suggests this phosphorylation may lie on its functional pathway (*11*).

A role for the nm23 gene in the development and differentiation process has been suggested by studies of its *Drosophila* homologue abnormal wing discs (awd), in which reductions in awd gene expression or mutation resulted in lethal abnormalities in cell morphology and differentiation postmetamorphosis (*12*). In mammals, increased Nm23 protein was detected immunohistochemically in virtually all epithelial tissues during mouse embryogenesis concurrent with their functional differentiation, although Nm23 protein was not maintained in all adult differentiated epithelia (*13*). Direct evidence of a role for Nm23 protein in mammalian differentiation is lacking to date. We report the first functional evidence of a role for nm23-H1 gene overexpression in the morphological and biosynthetic differentiation of a human breast carcinoma cell line in response to culture within a reconstituted basement membrane. The data implicate a novel role for the nm23 gene in breast physiology and strengthen the hypothesis that tumor metastasis and embryogenesis may use similar or identical genetic pathways.

Materials and Methods

Cell culture. Human metastatic MDA-MB-435 breast carcinoma cells, derivatives transfected with the control vector pCMVBamneo (C-100 and C-103), and derivatives transfected with pCMVBamneo-nm23-H1 complementary DNA (cDNA) [H1-170 and H1-177, expressing fourfold and eightfold greater Nm23 protein than the control vector cell lines, respectively, (*9*)] and normal HMT-3522 breast epithelial cells were cultured as previously described (*1,9*). EHS matrix was prepared from EHS ascites tumors passaged in C57BL mice at a concentration of 7-10 mg/mL and stored at 0 °C for up to 4 weeks as described (*1*). The care of the C57BL mice was in accord with institutional guidelines. In some experiments, commercially prepared EHS-matrix (Matrigel; Collaborative Research Inc., Bedford, Mass.) was used. Prior to seeding into EHS matrices or Matrigel, MDA-MB-435 cells and transfectants were cultured for 48 hours on collagen type I-coated plates in serum-free CDM3 medium as described previously (*1*). The cell lines were trypsinized and replated either into monolayer culture or into 300 μL of EHS matrix or Matrigel (7-10 mg/mL) as single cells at a concentration of approximately 2.5×10^5 cells per well of a 24-well plate and cultured as described previously (*1*).

Immunochemistry. Frozen and formalin-fixed, paraffin-embedded sections (5 μm) were prepared from EHS cultures. The Nm23 protein was localized in paraffin sections and Bouins-fixed monolayer cultures using affinity-purified anti-Nm23 peptide 11 antibody (*12*). Frozen sections were used for localization of 1) milk-fat globule membrane antigen (MGFGM-A; sialomucin) using monoclonal antibody 115D8 (San Bio, Am Uden, The Netherlands) as described (*1*); 2) cadherins with anti–P- and anti–E-cadherin antibodies (gifts of Drs. S. Hirohashi and C. Damsky); 3) type IV collagen with antibody PHM-12 (AMD; Armaton, New South Wales, Australia) and COP (Medac, Hamburg, Federal Republic of Germany); and 4) laminin with antibody M638 (Dakopatts, Glostrup, Denmark). Control sections were stained with secondary antibodies only.

Growth analysis. Colony cell content was determined microscopically as described (*1*). Thymidine labeling indices were determined by 24-hour incorporation of [^{3}H]thymidine (20 Ci/mmol; Du Pont NEN Research Products, Boston, Mass.) as described (*1*). To ensure that cells were at comparable densities at the time of thymidine labeling index determination, Nm23 protein-positive clones were seeded within EHS at five times the concentration of control or parental cultures. For monolayer culture, the cells were plated at 2×10^4 cells/cm^2 and labeling indices were determined on day 12 of culture at subconfluent densities.

Statistical analyses. The two-tailed Student's *t* test was used to compare cells-per-colony data and sialomucin production. Analysis of the time course of basement membrane formation/[^{3}H]thymidine labeling was performed using the nonparametric Wilcoxon signed rank test.

Results

Analysis of Morphology of MDA-MB-435 Breast Carcinoma Cells Overexpressing the nm23-H1 Gene in a Three-Dimensional Matrix

The normal breast culture HMT-3522 was previously reported to form organized acinar structures when cultured within an EHS matrix, while CAMA-1, T47D, BT-20, ZR-75, HMT-3909, and MCF-7 breast carcinoma cell lines failed to recapitulate this process (*1*). To determine the effect of nm23-H1 gene expression on the morphological differentiation of breast cells, MDA-MB-435 breast carcinoma clonal cell lines transfected with pCMVBamneo vectors (C-100 and C-103) or the same vector containing the full length nm23-H1 cDNA (H1-170 and H1-177) were cultured within an EHS matrix for 12 days. Cultures were examined for Nm23 protein expression by immunohistochemistry and for the presence of acinus-like structures by microscopy. Both the parent MDA-MB-435 cell line as well as the control clones expressed little Nm23 protein and produced large disorganized colonies (Fig. 1, D and F). In contrast, the H1-177 transfectant expressed significant Nm23 protein and produced small spheres with occasional lumens or remained as single cells (Fig. 1, B). The morphology of two different passages of the H1-170 cell line was heterogeneous, containing nm23-negative colonies of unorganized morphology as well as nm23-positive spherical colonies (not shown). The morphology of the H1-177 cells and Nm23 protein-positive H1-170 cells differed from that of the previously characterized HMT-3522 cells (*1*) only in the proportion of spheres with a central lumen. Less than 1% of the Nm23 protein-positive spheres contained a lumen.

Analysis of Basement Membrane Deposition

In addition to morphological evidence of differentiation, culture of normal HMT-3522 cells within an EHS matrix induced the expression of basement membrane proteins and their deposition to the outside of the acinus-like spheres (*1*). Analysis of six breast carcinoma cell lines and two primary carcinoma cultures failed to show similar trends (*1*). Immunohistochemical staining of type IV collagen and laminin was conducted on parental MDA-MB-435 cells, control transfectants, and nm23-H1 gene transfectants cultured within an EHS matrix (Fig. 2 and data not shown). By day 6 of culture, 87.8% ± 3.8% (means ± SE) of Nm23 protein-positive transfectants deposited type IV collagen; this percentage increased to 97.1% ± 1.9% by day 12 of culture. These latter data compared closely with the normal HMT-3522 cultures, in which 100% of spheres deposited a basement mem-

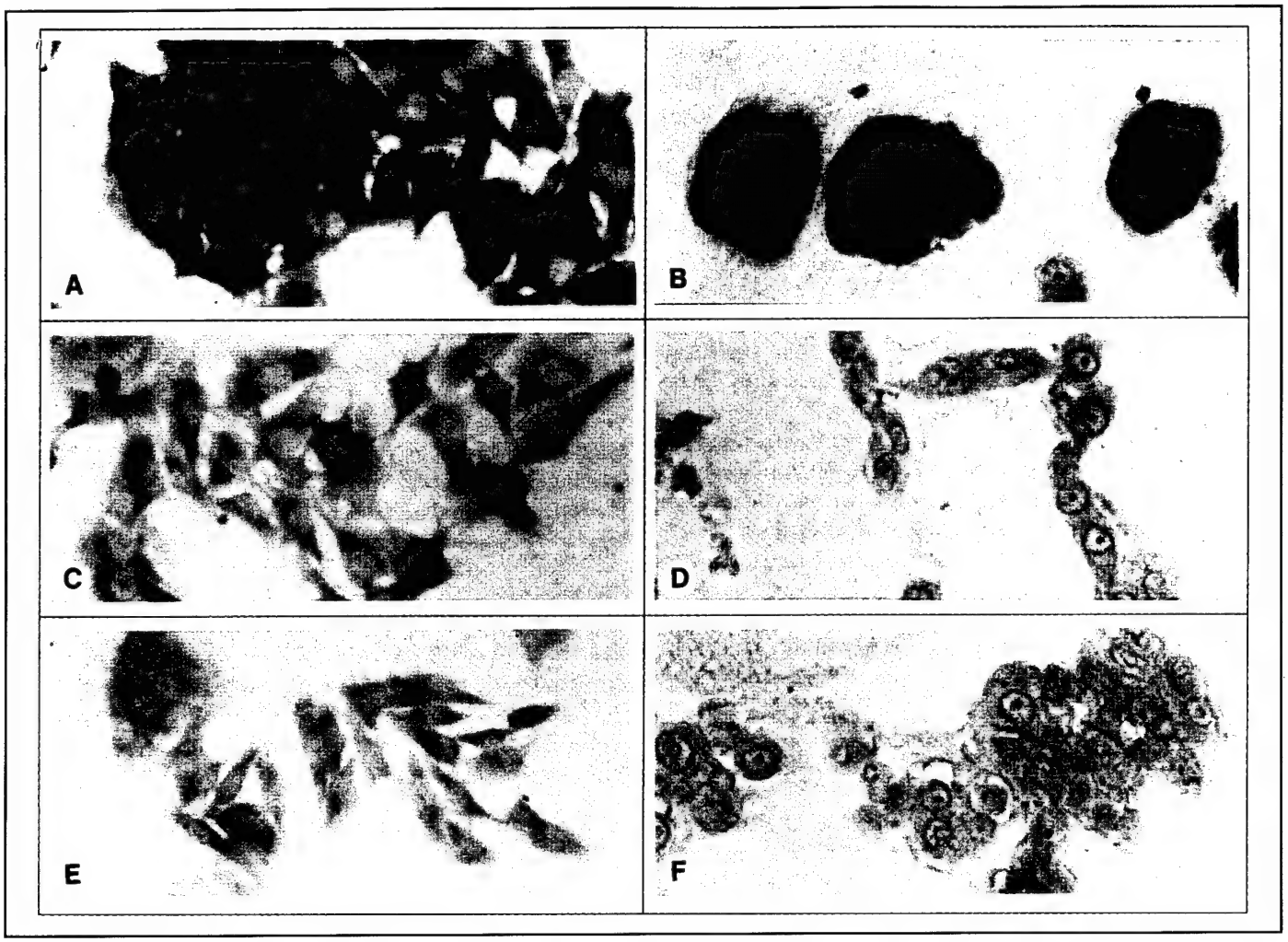

Fig. 1. Immunohistochemical staining of Nm23 protein to show morphology and Nm23 protein expression in 12-day cultures of MDA-MB-435 cells in monolayer (**A**, **C**, and **E**) and in EHS matrix (**B**, **D**, and **F**). Panels **A** and **B** show nm23-H1 gene-transfected clone H1-177; Panels **C** and **D** show control-transfected clone C-100; panels **E** and **F** show untransfected parent MDA-MB-435 cells. Note the intense cytoplasmic staining for exogenous Nm23 protein in H1-177 cells (**A** and **B**) and the capacity of these cells to form spheres in EHS (**B**). Panels **A**, **C**,and **E** (original magnification ×440; panels **B**, **D**, and **F** original magnification ×550).

brane by day 12 of culture. However, the basement membrane of HMT-3522 cells differed from nm23 gene transfectants in that it was detected with a broader range of anti-type IV collagen antibodies (data not shown), suggesting differences in basement membrane immunoreactivity. Deposition of laminin, although occasional, weak, and fragmented compared with type IV collagen, confirmed the presence of an elaborate basement membrane in nm23 gene transfectants (Fig. 2, A, inset). Basement membrane proteins were localized to the outside of the acinus-like spheres. In contrast, none of the parental cells, control transfectants, or Nm23 protein-negative H1-170 transfectants expressed type IV collagen or laminin.

Other Biosynthetic Activities

Immunostaining was conducted for sialomucin, a glycoprotein synthesized by epithelial cells that accumulates at the apical cell surface of polarized mammary epithelia and in milk-fat droplets. Increased staining of sialomucin was observed in Nm23 protein-positive cells compared with control transfectants (Fig. 3). By day 9 of culture, 49.5% ± 4.5% of Nm23 protein-positive transfectants expressed sialomucin compared with 5.5% ± 1.5% of controls ($P = .011$). Deposition of sialomucin was apical and lateral in normal HMT-3522 cultures and nonpolar in Nm23 protein-positive transfectants. The frequency of sialomucin expression was lower than that of basement membrane deposition for both Nm23 protein-positive MDA-MB-435 and HMT-3522 cells, where 12% of spheres expressed sialomucin and 100% expressed basement membrane (*1*).

The potential role of cell–cell adherence in acinar differentiation in culture was determined. While HMT-3522 cells exhibited basolateral deposition of E- and P-cadherins, the parental MDA-MB-435 cells and all transfectants failed to express detectable protein at the cell membrane (data not shown). Thus, the morphological and biosynthetic evidence of differentiation occurred independently of E- and P-cadherins.

Formation of Acinar Structures by the nm23-H1 Gene Overexpressing MDA-MB-435 Breast Carcinoma Cells: Association With Growth Inhibition

Monolayer culture of the control- and nm23-H1–transfected MDA-MB-435 breast carcinoma cell lines on tissue culture

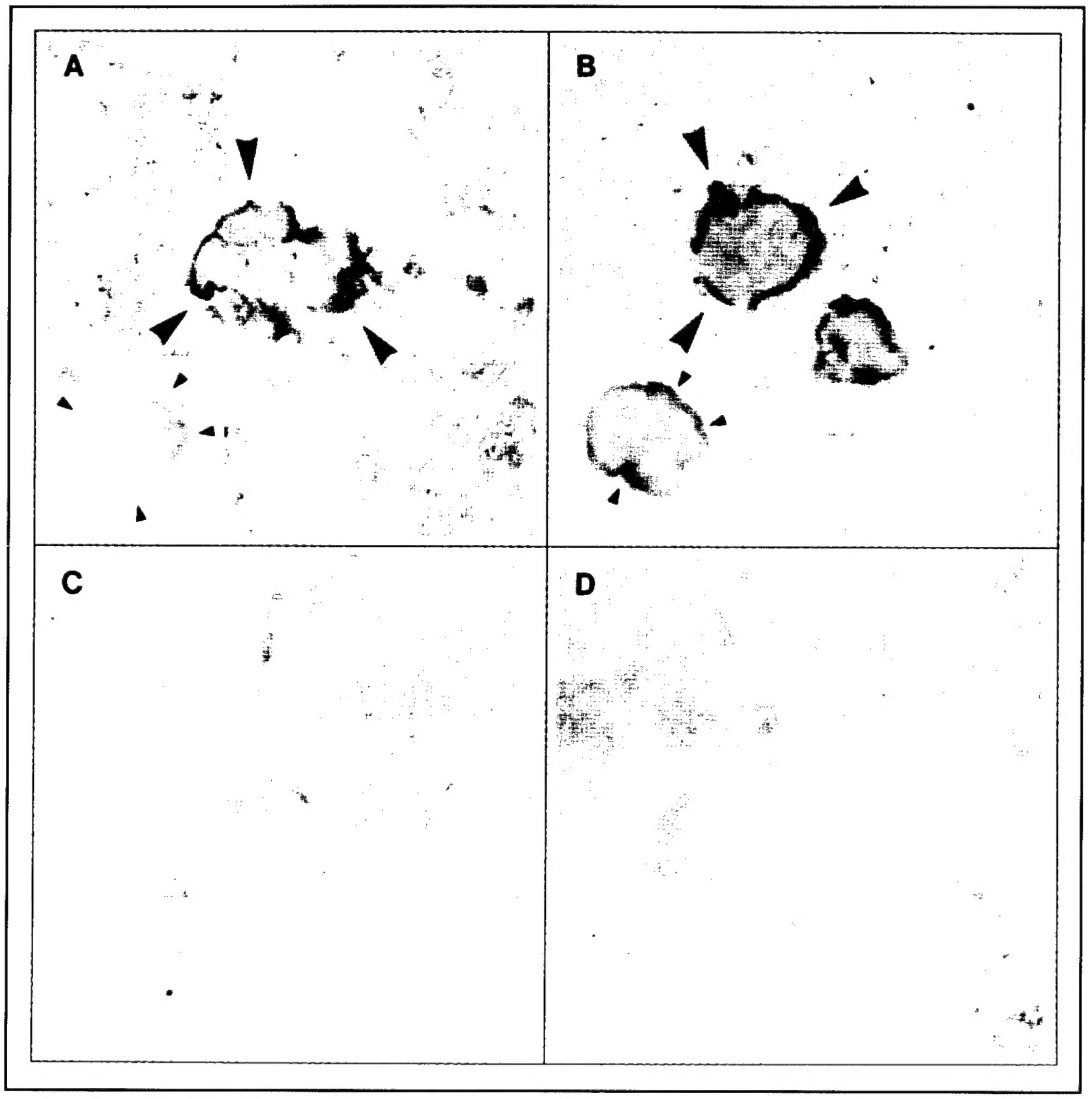

Fig. 2. Immunohistochemical staining of type IV collagen and laminin expressed by nm23-H1 gene-transfected MDA-MB-435 clone H1-177 cells (**A**) and normal HMT-3522 breast epithelial cells (**B**). Arrows show localization of type IV collagen at the basal surface of spheres formed by nm23 gene-transfected cells and normal breast cells. Insets show similar localization but less intense staining of laminin for H1-177 cells (inset of panel A) and for reference HMT-3522 cells (inset of panel B). Note the absence of collagen IV deposition by the untransfected parental MDA-MB-435 cells (**C**) and control transfectants clone C-100 (**D**) (original magnification ×400; inset original magnification ×320).

plastic revealed no significant differences in growth rate. Thymidine labeling indices conducted in the passages of these cell lines used in the present series of experiments were 98.2 ± 0.9, 96.8 ± 0.8, 96.9 ± 0.8, and 98.5 ± 1.2 for the C-100, C-103, H1-170, and H1-177 cell lines, respectively. We initially noted a difference in both the morphology and size of colonies produced by control and nm23-H1 gene transfectants when cultured within an EHS matrix. Table 1 lists the number of cells per colony in parental, control, and nm23-H1 gene-transfected MDA-MB-435 cells on day 12 of culture within an EHS matrix. The parent line and control transfectants produced colonies ranging from 16-26 cells per colony. H1-177 cells produced colonies containing an average of 9.6 cells ($P<.001$), which compared closely with the 8.0 cells per sphere exhibited by normal HMT-3522 cells. Analysis of H1-170 cells further strengthened this trend; Nm23 protein-negative colonies contained a mean of 27 cells, while Nm23 protein-positive colonies contained a mean of 8.2 cells ($P<.001$). Similar trends were observed on culture of the control- and nm23-H1–transfected cell lines within Matrigel (data not shown).

The relationship of biosynthetic and growth inhibition aspects of breast cell differentiation have been evaluated in the H1-177 cell line. Table 2 shows the time course of basement membrane deposition and thymidine labeling in the H1-177 cell line. Deposition of basement membrane was prevalent among colonies by day 6 of culture (87.8%) and virtually homogeneous by day 12 of culture (97.1%). In contrast, the percentage of thymidine labeled cells remained high at day 6 of culture (92%) but was reduced to 29.9% by day 12. Evaluation of both parameters simultaneously for H1-177 cells is also shown. The percentage of spheres that were basement membrane positive–[^{3}H]thymidine negative rose from 7.6% on day 6 of culture to 70.1% on day 12 ($P = .001$). A concurrent decrease in the percentage of basement membrane-positive–[^{3}H]thymidine-positive spheres was observed, from 80.2% on day 6 to 27.0% on day 12 ($P = .002$). All the remaining basement membrane-negative spheres were [^{3}H]thymidine positive on day 12 of culture. The data suggest the hypothesis that basement membrane synthesis and secretion, an early event in this system, may signal an inhibition of cell growth. Taken together, the

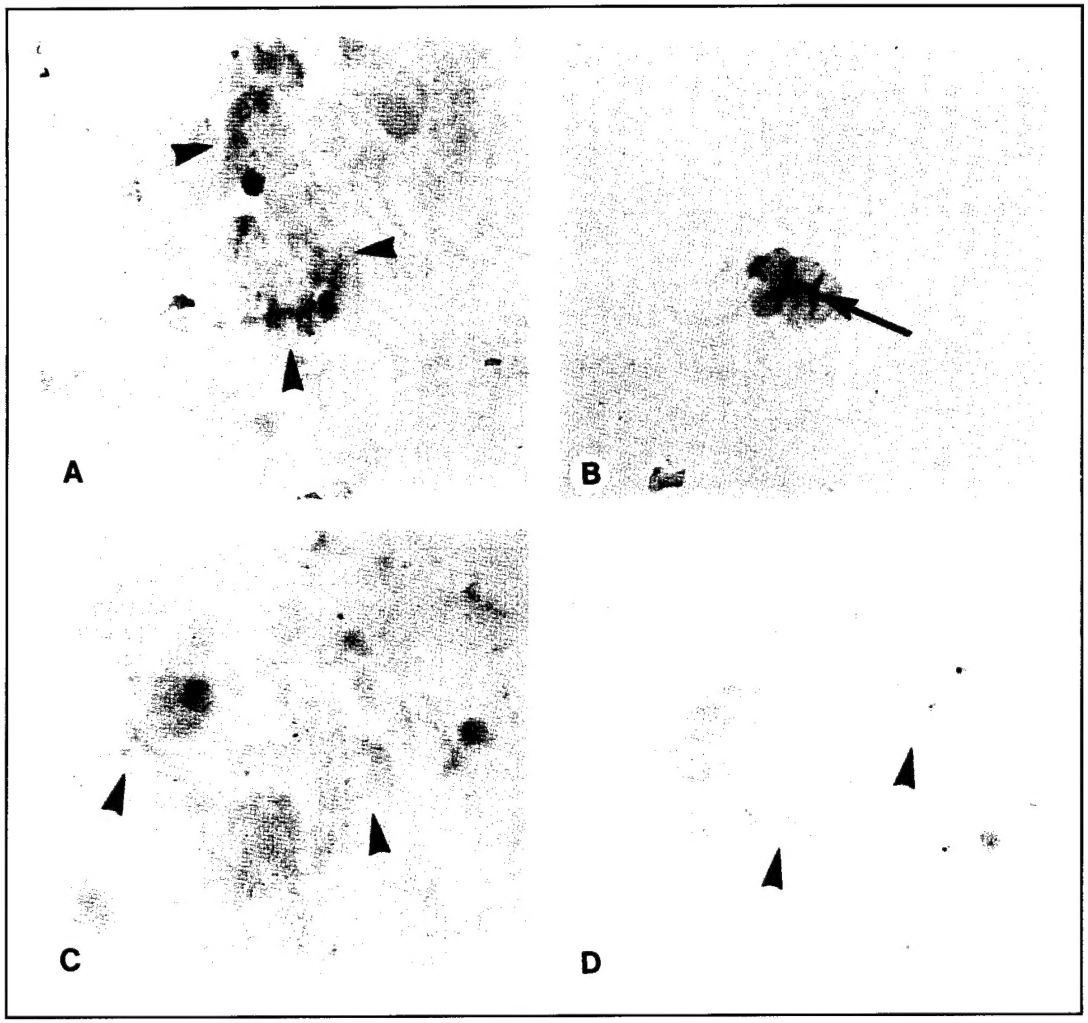

Fig. 3. Immunohistochemical localization of sialomucin (arrows) expressed by MDA-MB-435 clone H1-177 cells (**A**), normal control HMT-3522 cells (**B**), control MDA-MB-435 clone C-100 cells (**C**), and control untransfected parental MDA-MB-435 cells (**D**). Note the apical and lateral accumulation of sialomucin by normal HMT-3522 spheres (**B**) and the nonpolar expression of sialomucin by the MDA-MB-435 cells (**A**, **C**, and **D**) (original magnification ×400).

data provide evidence of an antiproliferative effect of nm23-H1 gene expression in breast epithelial cells.

Discussion

The importance of lactogenic hormones and basement membrane for morphological, biosynthetic, and growth regulatory aspects of mammary differentiation is well established for cells of both rodent and human origin. Culture of dispersed rodent mammary epithelial cells [(*14*,*15*), reviewed in (*16*)] or human reduction mammoplasty cultures (*1*) on or within basement membrane components has resulted in the formation of polarized acinar structures, production and vectorial secretion of milk proteins, and limited growth rates. Yet, under similar conditions, evidence of differentiation was lacking when primary cultures of human breast carcinomas or human breast carcinoma cell lines were used (*1*). These observations indicate the importance of additional, unknown factors intrinsic to breast cells in the differentiation process.

The present article has evaluated the potential contribution of nm23-H1 gene expression on morphological, growth regulatory, and biosynthetic aspects of breast differentiation, using the metastatic human MDA-MB-435 breast carcinoma cell line cultured within a physiologically relevant microenvironment of reconstituted basement membrane. Two lines derived from the metastatic human MDA-MB-435 breast carcinoma cell line, which overexpress the nm23-H1 gene, recapitulate portions of the breast differentiation process in reconstituted basement membrane culture, including the formation of acinus-like structures, directional deposition of basement membrane components, expression of sialomucin, and limitation of growth. These observations are in contrast to the behavior of the parental MDA-MB-435 cell line, the C-100 and C-103 control transfectants, and revertants of the H1-170 cell line that do not overexpress the nm23-H1 gene. The results represent the first cause and effect data concerning nm23-H1 genes and mammalian differentiation. The data do not imply that nm23 gene expression universally controls breast differentiation; the inability of tumor cell lines such as MCF-7 to fully differentiate in this system, despite relatively high expression of the nm23 gene (*17*), confirms the existence of additional important regulatory events. However, the statistically significant correlation of high Nm23 protein expression with a high grade of differentiation in several infiltrating ductal breast carcinoma cohorts (*3*,*5*) suggests that data generated in this culture system may be relevant in vivo.

In previous experiments, the differentiated phenotype of reduction mammoplasty cultures was correlated with full polarization of sialomucin secretion and correct cadherin mediated

Table 1. Overexpression of the nm23-H1 gene by MDA-MB-435 human breast carcinoma cells: association with the formation of smaller colonies upon culture within EHS matrix on day 12

Cell line	Mean ± SE cells per colony*	P†
HMT-3522	8.0 ± 0.3	
MDA-MD-435 parental	19.1 ± 0.9	
C-100	16.5 ± 0.8	
C-103	25.8 ± 1.4	
H1-170 (nm23 negative)	27.3 ± 2.1	
H1-170 (nm23 positive)	8.2 ± 0.6	<.001
H1-177	9.6 ± 0.2	<.001

*The number of cells per colony profile were counted in sections of EHS gels as described previously (*1*) (n = 20 profiles per point).

†Student's *t* test versus smallest control clone (C-100).

Table 2. Relationship between cell growth and basement membrane deposition in culture of the nm23-H1 gene transfectant H1-177 cell*

	% colonies ± SE	
Phenotype	Day 6 of culture	Day 12 of culture
[^{3}H]Thymidine positive	92.0 ± 2.5	29.9 ± 8.9
Basement membrane positive	87.8 ± 3.8	97.1 ± 1.9
Basement membrane positive/ [^{3}H]thymidine positive	80.2 ± 4.2	27.0 ± 7.4†
Basement membrane positive/ [^{3}H]thymidine negative	7.6 ± 2.6	70.1 ± 8.9‡
Basement membrane negative/ [^{3}H]thymidine positive	11.7 ± 3.9	2.8 ± 1.9
Basement membrane negative/ [^{3}H]thymidine negative	1.0 ± 0.9	0

*Basement membrane deposition was visualized by immunoperoxidase immunostaining, and DNA synthesis was determined by [^{3}H]thymidine autoradiography in sections of EHS cultures. Colonies were simultaneously scored for the presence of basement membrane and thymidine-labeled nuclei on the days of culture noted.

†Comparison of day 6 and day 12 using Wilcoxon signed rank, $P = .002$.

‡Comparison of day 6 and day 12 using Wilcoxon signed rank, $P<.001$.

expression [(*1*) and Bissell MJ and Petersen OW: unpublished observations]. Our data indicate that MDA-MB-435 cells can recapitulate certain portions of the differentiation process in the absence of detectable E- and P-cadherin expression at the cell membrane and without the polarized luminal expression of sialomucin.

Our data also indicate that nm23-H1 gene expressing colonies were composed of twofold to threefold fewer cells than control colonies by day 12 of culture (Table 1). Similar trends were observed using thymidine labeling and cell per culture measurements (data not shown). These data indicate a suppressive effect of nm23-H1 gene expression on cell growth in this system. These data stand in agreement with the inhibition of soft agar colonization in response to TGF-β among nm23 gene-transfected breast carcinoma and murine melanoma cells (*9,18*). However, they contrast the lack of significant differences between the control and the nm23 gene transfectants in primary tumor size for these lines in vivo (*9,18*) as well as their growth rates (*9,18*) and thymidine labeling indices (data presented herein) on tissue culture plastic. In addition, the expression of the nm23 gene in cohorts of infiltrating ductal carcinomas has not been significantly correlated with primary tumor size, where analyzed (*3,5,6*). There are several possible reasons for these apparently conflicting data. First, we have noted that the directional deposition of the basement membrane component type IV collagen preceded growth arrest in the nm23-H1 gene-positive H1-177 cell line (Table 2). These data permit the hypothesis that cellular contact with basement membrane in nm23-H1 gene-positive cells may constitute part of the signal transduction pathway involved in growth suppression. It is possible that, in primary tumors, the wealth of tumor and stromal cells may produce sufficient proteases to disrupt this interaction; in developing metastases where single tumor cells or small emboli are present, sufficient intact basement membrane may be formed to inhibit growth, thus contributing to the apparent lack of metastases. Second, growth stimuli overriding basement membrane-induced control of nm23-H1 gene-positive breast cell proliferation may exist in the mammary environment, such as locally produced growth factors and/or tumor cell–stromal interactions. The observations presented here, linking endogenous basement membrane deposition to the reversion of the highly metastatic MD-MB-435 cell line to a more normally differentiated phenotype, suggest that the presence of an intact basement membrane may be one critical point of regulation that is lost in malignancy. The present model promises to allow the elucidation of aspects of the molecular mechanisms involved in basement membrane formation and its relationship to growth arrest and will help to establish the significance of Nm23 protein in these processes.

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Notes

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EXCHANGE PROGRAM

European Organization for Research and Treatment of Cancer

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European awardees will receive the U.S. contribution either from the NCI or from their extramural host institution. The European contribution of the exchangeship will be provided either by the scientist's home institution or by a European granting agency.

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The following documents are required, in English, from all applicants:

- Completed application form.
- Description of the research to be undertaken, not to exceed three typewritten pages.
- Letter of invitation from the prospective host.
- Agreement to release the applicant from the home institution for the duration of the exchangeship.
- Assurance of intention to return to the home institution at the end of the exchangeship.
- Statement concerning the provision of 50 percent of financial support by European sources. Non-EORTC member country candidates must continue at full salary at the home institution for the duration of the exchangeship.
- Three letters of recommendation mailed directly to the NCI Liaison Office by the recommending individuals.

For More Information Contact:

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